Biology I
LB-144
Cell and Organismal Biology

Lecture & Lab
Fall 2024
(for student in Douglas Luckie's sections)

This publication is a compendium of work authored by:
Candace Igert, Angela Wright, Cori Fata-Hartley, Michael Haenisch, Susannah Cooper, Marjia Krha, Joseph Maleszewski, John Wilterding, Mimi Sayed, and Douglas Luckie

Contents

Welcome to LB-144

Welcome to LB 144! The experience you are about to have in the cell & organismal biology course may turn out to be unlike most other lectures/labs that you will have while as an undergraduate. There will be a major emphasis on team effort in this class and your ability to work in a team is crucial to your success in this course and beyond. As a team, you and your partners, will work together to solve biological problems in lecture, discuss scientific ideas in recitation, and pursue research projects within the lab to find answers to the unique questions your group will have posed.

Molecular/Cell/Organismal biology is quickly becoming an integral part of science and society. With new discoveries and ongoing discussions of topics such as: cloning, genetically modified foods/organisms, DNA fingerprinting, genomics, bioinformatics, gene patenting (the list goes on) it becomes crucial to connect it all to the cellular and organismal levels.

Cell & Organismal biology is not an easy subject to master. There are complex concepts as well as a great deal of factual information. Nevertheless, the difficulty of this subject adds to its appeal. The staff of LB 144 will work hard to help you glean the information necessary to achieve in this course; however, your hard work is the most essential element to success.

This experience will without a doubt be exciting, frustrating, and almost overwhelming at times – but it is all with purpose. We hope that you will come out of this course with not only a better understanding of molecular biology, but also a better understanding of the scientific method itself.

We look very forward to working with you throughout the semester and if you have questions please do not hesitate to contact any one of the TAs, GAs, or the Professor. Good luck and enjoy the semester!

- The 144 Staff

144 Contract (sign & return)

A syllabus is a form of contract between the instructor and the students. If you, the student, complete tasks with a specific score a predefined grade is awarded. Read the announcements below and the syllabus *in full* before signing and submitting this page.

1. HYBRID *I am aware this course is hybrid; half the lectures are online and require TopHat.*

2. WORKLOAD *As an Undersigned student, I am aware the LB144 lecture & lab courses together are worth 4 credits and will require me to work outside of class 8-12 hours each week.*

3. TOURISM *I am aware that I will work with a group of students who will meet and study together at night and on weekends. If I need to travel frequently, I should discuss this with them.*

4. MANY READINGS *As the Undersigned student, I am aware that I will have required readings for both the lecture and lab courses each week, and unless I read the assigned pages, answer questions, take notes, and study them prior to class, it's likely I will become lost during class.*

5. QUIZZES *As the Undersigned student, I am aware that I may have quizzes or graded exercises each week, and unless I read the assigned pages in the reading, take notes and study them prior to the quiz, it's likely I will get a low score on the quiz or exercise.*

6. EXAMS *As the Undersigned student, I am aware exams may be purely open book essay-style and provided in advance, and in this case, I should work with my group studying the questions and developing excellent answers in the time prior to the test. If I just "cram" my studies and work into 48 hours prior to the exam, it's likely I will get a low score on the midterm.*

7. UNIVERSITY GRADING SCALE *I am aware this course uses a university scale with higher expectations than high school, excellent work is a 3.0, and much more is necessary for a 4.0.*

8. GROUP GRADES *I am aware that I, with the help of others, will be authoring one research paper (with several drafts) and my grade may include both the score of my sections as well as the score for the whole paper. I realize I will be expected to review the entire paper before submission. If this doesn't work well for me, I should discuss it with my group or the Prof immediately.*

9. HONOR CODE *In the authoring of assignments, I accept that any piece of work may be submitted to http://turnitin.com for screening. I am aware that if the work authored by me is found to be plagiarized, I will be given a zero for the assignment & perhaps for the LB144 course grade.*

10. LAB PARTICPATION *I am aware that in the laboratory course, each and every week, I am expected to go out in the field and collect observational data and record it in a handwritten notebook as well as find new research papers for my group project.*

I have read this contract, I understand, I'm up to the challenge, I agree to these tenets.

_____

Printed Name *Signature* *Date*

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Printed Name *Signature* *Date*

HYBRID EDITION: THIS COURSE WILL BE DELIVERED IN A HYBRID MODE.

LB-144: CELL & ORGANISMAL BIOLOGY

If you strive to think and communicate like a scientist in your work throughout the semester, in the end, you will be a scientist and really know biology (Think like a serious scientist, not like a pretend one).

LECTURER

Douglas B. Luckie (he/him/his), Professor, Lyman Briggs College & Department of Physiology
zoom Office: 5173534606, **luckie@msu.edu,** Holmes Office: W-26D, Phone: 517-353-4606

COURSE WEBSITE **http://ctools.msu.edu/144** (mirror => cf.psl.msu.edu/144)

TEXTBOOK (online-only, for Luckie's LB144 custom textbook, http://store.trunity.com/)
"Integrating Concepts in Biology" by Campbell, Heyer & Paradise, 2024 Edition, Trunity Holdings Inc

COURSE PACK "LB-144 Course Pack," (Luckie) from MSU Library Services via local bookstore

INTERNET 20+Mbps (download): 10+Mbps (upload) best. Use D2L, Zoom, TopHat, CATME, Turnitin.

LB-144 = LB-144 (LECTURE) & LB-144L (LAB) OVERVIEW OF CLASSES
There are two overreaching goals in these two connected classes.

1.) To gain a fundamental comprehension of the cellular & organismal processes of life and an appreciation why it is important to understand these processes.
2.) To learn how to think like a scientist and be able to adaptively negotiate a question or problem.

The cell & organismal biology course is an exploration of life at all levels. It examines the interplay of genes, cells, and chemistry allowing organisms to live, survive, and interact with each other and the environment. Specifically, we will study genes (molecular biology), living cells inside organisms (cell biology), organisms and their environments (ecology), genetic variation and inheritance (genetics), and the interactions of the environment and genetics over time (evolution) that led to the diversity of life observed on the planet today.

Our lecture will meet twice a week as two 80-minute class meetings. Once a week in-person (live, synchronous) and once on TopHat (recorded, asynchronous). In this class, you will have daily homework and in lecture we will review and discuss, in depth, parts of the readings. We will examine how scientists perform their work to help you master the ability to ... *think like a serious scientist, not like a pretend one*.

WORKLOAD
The first semester of LBC Biology, LB144, consists of two connected courses (lecture 3 credits, laboratory 1 credit) and because LB144 is two classes they require twice as many hours of work as one class. For any university-level course, for each credit, you are expected to spend 2-3 hours/week outside of class studying and working on homework assignments. There will be a certain amount of preparation that you will need to do before each lab and readings that you will need to complete (with notes taken) before each lecture. Come to lecture and lab well-prepared or mastering the new material may be far more difficult and stressful.

SCHEDULE
Both the lecture schedule and the lab schedule are found in the two syllabi provided. We reserve the right to modify schedule if necessary. You will be given advance warning if the schedule needs changed.

OFFICE HOURS & JCLUB
Held each week on Mondays 10:20-11:40am in-person in our classroom & you can make appointments.

ACADEMIC HONESTY
To avoid problems ***turnitin.com*** will allow you to upload and review writing assignments prior to submission for grading. Yet, if you are caught cheating, you will be assigned a "0" for the assignment or the course. The LBC academic honesty policy is -> https://lbc.msu.edu/advising/academic-policies.html

GRADING
Your grade in LB144 is based on the total percentage earned in the both the lecture course and the laboratory course, each worth half. Your work will be graded on a flat scale.

4.0= 90-100% 3.5= 85-89.9% 3.0= 80-84.9% 2.5= 75-79.9% 2.0= 70-74.9% 1.5= 65-69.9% 1.0= 60-64.9% 0.0= <60

A "3.0" score is considered Excellent. It is impressive work, top of the class, and the work was done extremely well but nothing beyond what was expected.
A "3.5" is Most Excellent. Every detail of the work was done extremely well, and they found additional papers and evidence beyond what they were required.
A "4.0" is Outstanding. It has the 3.0, 3.5-level elements + student impresses instructor with how much/well they used additional papers and evidence.

Late Policy: Assignments are due in lab/lecture at the beginning of the session indicated (at the time of entering the room) unless otherwise specified. If an assignment is 1 day late, only 1 point will be deducted from the final score. After this 24-hour grace period, the penalty becomes more severe: 20% off for two days late, 30% off for three days, and so on. After 5 days, you will receive a "0" for the assignment.

Rejected Manuscripts/Reports: Each time a paper is "rejected", because it did not follow the *Instructions to Authors*, 1 point is deducted. This is independent of the Late Policy, both can occur.

Blind grading: Whenever possible we will score assignments "blind" and thus ask you to not list your name but your "B-PID" (found in D2L grade book). This helps eliminate bias and makes grading more fair.

***Formal written grade appeal process**: If you feel that your assignment was not graded properly, you may submit an appeal in writing (on paper, not via email). You must concisely explain why and how your work in fact was correct and demonstrated you mastered that element of the grading rubric, providing sources. Please be advised that if you submit a formal grade appeal about one part of an assignment, we always re-grade your entire exam, paper, or quiz and the score may increase, decrease, or stay the same. For group assignments, all authors must sign the written request since re-grading may impact all. How well you provide your claim, evidence, and reasoning will be assessed, and students who provide good logical succinct arguments supported well by solid relevant evidence will earn approval (you may cite pages of textbooks, or even better, published research papers). Avoid emotional arguments that blame others or arguments based on hearsay, e.g. "I heard from a student" "A TA told me this was correct." If you do not make logical arguments or provide thoughtful evidence to support them, your appeal will not gain traction to be approved. All discussion concerning score changes must be completed within 7 days from the date the grade was officially posted (on the returned assignment or online). No grade changes will be considered after this time. If illness or other emergency prevents you from completing assignments on time, you must make arrangements with your instructor before the due date (*example* of appeal provided in course pack).
TIPS: Explicitly list/label "Claim" "Evidence" "Reasoning" on your appeal. Clearly identify which specific element of the grading rubric you are appealing. Text should be concise, a single-page appeal is optimal.

LB144 Biology Learning Goals

1. Practice at doing and communicating science. Speak your thoughts smartly.

a) Communicate Scientific Thoughts: Manifest your smart thinking in the best words possible.
   1. Speaking: a high priority of this course is for you to practice public speaking & listening.
   2. Reading: practice careful reading of papers, identification of points, interpretation of figures.
   3. Writing: practice composition of text, writing manuscripts, building data figures and graphs.

b) Design and Analyze Experiments: Make a hypothesis, design experiments, make predictions. Interpret data collected, look for patterns, ways to best share and represent findings.

2. Study the Biology Idea of "Information". Learn examples and mechanisms to store/transmit information at molecular, cell, organismal, population levels.

These "**content**" goals are for you to understand, describe, and give examples of how:

a) Heritable information (like DNA/genes) provides for continuity of life and non-heritable information (like talking) is also transmitted within and between biological systems.

b) Imperfect information transfer, like during reproduction of cells, chromosomes, and genes, leads to *variation* of traits among individuals. (e.g., some beach mice have light colored fur because a mutation in a gene makes it difficult for their hair cells to make dark hair pigment)

c) Interactions among organisms and the environment determine *individual* survival and reproduction. (e.g., animals who are in cooperative groups and communicate live longer)

d) Selection (and other mechanisms) acts on individuals and leads to the evolution of *populations*. (e.g., beach mice with fur that matches the color of sand live longer than others because?)

e) Information in DNA => becomes (transcribed) information as RNA => becomes (translated) information as proteins (e.g. How viruses enter our cells, take control, & make viral proteins)

f) Small simple chemicals can associate to form nucleotides, amino acids, lipids, carbohydrates; which can polymerize, form structures and functions we define as "alive" (life on earth).

3. Practice Transfer of Learning: Work with your group to intentionally transfer knowledge learned in one context (e.g. squirrels) to another new context (e.g. humans).

a) Reflect: Develop personal learning goals and regularly reflect on your progress during the semester. (e.g. regularly consider "What I am supposed to be learning here? Have I mastered that? What about transfer? Can I link this to life on Mars, or humans, or something different?")

b) Collaborate: Confidently cooperate in teamwork, and practice team building, communication and leadership. (e.g. "that's a good idea, should we also test if it works in another animal?" "Jon, you haven't spoken much, what do you think?")

HYBRID EDITION

LB-144: CELL & ORGANISMAL BIOLOGY (LECTURE)

SCHEDULE: Each week= TOP HAT online & *LIVE in-person*

	Date	*Scale/Level*	*Readings (emphasis)*	*Instructors LIVE or TopHat*
W1	W, 28 **Aug.**	Ecological	Lect. 1, Ch 18.1 (crickets call)	*LIVE in-person (Luckie & LAs)*
W2	online	Ecological	Lect. 2, Ch 18.1 (frogs sing)	Online videos (Luckie & LAs)
	W, 4 **Sep.**	Ecological	JClub1: Ulagaraj+Page papers	*LIVE in-person (Luckie & LAs)*
W3	W, 11	Population	Lect. 3, Ch 18.3 (corals settle)	*LIVE in-person (Luckie & LAs)*
W4	online	Population	Lect. 4, Ch 17.1&.2 (fireflies)	Online videos (Chris Paradise)
	W, 18	Population	JClub2: Lewis+Harrington papers	*LIVE in-person (Luckie & LAs)*
W5	W, 25	Population	Lect. 5, Ch 17.2 (storm petrel)	*LIVE in-person (Luckie & LAs)*
W6	online	Population	Lect. 6, Ch 17.3 (meerkats)	Online videos (Chris Paradise)
	W, 2 **Oct.**	Population	JClub3: Bretagnole+Manser papers	*LIVE in-person (Luckie & LAs)*
W7	online	Organismal	Lect. 7, Ch16.1 (Sandworts)	Online videos (Chris Paradise)
	W, 9	Organismal	JClub4: Caiazza et al paper	*LIVE in-person (Luckie & LAs)*
W8	M, 14	***EXAM I***	*LIVE in-person (in classroom)*	
	W, 16	***EXAM I***	*LIVE in-person (in classroom)*	

Fall Break, Oct. 21-22

W9	online	Molecular	Lect. 8, Ch1.1, 1.2 (Griffith)	Online videos (Luckie & LAs)
	W, 23	Molecular	JClub5: Watson+Crick papers	*LIVE in-person (Luckie & LAs)*
W10	online	Molecular	Lect. 9, Ch1.5 (Epigenetics)	Online videos (Malcolm Campbell)
	W, 30	Molecular	JClub6: DeSimone paper	*LIVE in-person (Luckie & LAs)*
W11	online	Cellular	Lect. 10, Ch2.3 (Translation)	Online videos (Malcolm Campbell)
	W, 6 **Nov.**	Cellular	JClub7: Dang+Johnson papers	*LIVE in-person (Luckie & LAs)*
W12	online	Organismal	Lect. 11, Ch3.1 (Mendel)	Online videos (Malcolm Campbell)
	W, 13	Organismal	JClub8: Collins papers	*LIVE in-person (Luckie & LAs)*
W13	online	Molecular	Lect. 12, Ch4.1 (Evolution)	Online videos (Malcolm Campbell)
	W, 20	Molecular	JClub9: Ingman et al paper	*LIVE in-person (Luckie & LAs)*
W14	online	Molecular	Lect. 13, Ch4.3 (Competition)	Online videos (Malcolm Campbell)
	W, 27	Molecular	JClub10: Miller paper	*LIVE in-person (Luckie & LAs)*

Thanksgiving Break, Nov. 28-29

W15	M, 2 **Dec.**	***EXAM II***	*LIVE in-person (in classroom)*
	W, 4	***EXAM II***	*LIVE in-person (in classroom)*

FINAL EXAM finals week 2024, see official schedule (tentative time/date)

THE LECTURE
ASSIGNMENT SCHEDULE

Researchers have found increased structure and active learning increase everyone's ability to learn in introductory biology courses[1]. In addition, every student in our course really does want to have time to slowly carefully read the textbook, learn new information and enjoy mastering topics in biology. Given we believe the textbook we are using is outstanding, we are only assigning short readings, with integrating questions, so you have adequate time to carefully read each section and reflect upon it. A quiz or exercise based on the reading may be given during each lecture. These quizzes/exercises are designed to help you assess your own learning before and between exams. They provide you with regular feedback as to how well you are mastering each topic.

ATTENDANCE AND PARTICIPATION IN LAB & LECTURE*:* It is essential that you not only come to class but also actively participate in order to construct your own knowledge. While *attendance* is being physically "present", *participation* includes reading and preparing well for class, answering questions verbally, and via clicker questions, collecting data in the field and recording it daily in your lab notebook. Active participation includes, the following behaviors:

1. *Bringing forth new ideas, information, or perspectives to academic conversations*
2. *Discussing your readings and reflections with instructors and peers*
3. *Meeting with the instructors to discuss your interests, assignments, or project*
4. *Participating in small group discussions and activities*
5. *Assuming responsibility for personal behavior and learning*

While working on group projects, students should be mindful, all participants should exercise:

- *Respect for themselves, each other*
- *Openness and a positive attitude toward new ideas and other's ideas*
- *Flexibility and tolerance of ambiguity*
- *Good communications amongst themselves.*

EXAMS*:* There will two exams and a final exam, each may be comprehensive of all prior material. Midterm exams may be traditional multiple-choice format, or may be essay-style Answers to open-book & take-home exams must also be submitted online to http://turnitin.com/.

Assignments (pts)**:**

Week	**Assignment(s)**	**@Lecture**	**%**
(*all*)	Attendance, Participation, Homework, Quizzes	X	**10**
5	**Exam I**	**X**	**30**
10	**Exam II**	**X**	**30**
15	**Final Exam**	**X**	**30**

Total = 100% of lecture grade

[1] Haak, D., J. HilleRisLambers, E. Pitre, and S. Freeman. 2011. Increased structure and active learning reduce the achievement gap in introductory biology. *Science* 332:1213-1216. Freeman, S., D. Haak, and M.P. Wenderoth. 2011. Increased Course Structure Reduces Fail Rates in Biology. *CBE Life Science Education* 10 (2):175-186

HYBRID EDITION

LB-144: CELL & ORGANISMAL BIOLOGY (LABORATORY)

Lab will meet on both Mondays and Wednesdays

LAB COORDINATOR

Douglas B. Luckie, Ph.D., Professor, Lyman Briggs College & Department Physiology

LAB MANUAL

found inside "LB-144 Course Pack," (Luckie) from MSU Library Services via local bookstore

COURSE WEBSITE http://ctools.msu.edu/144

RESEARCH TEAM RATIONALE

Student groups are intended to be research & learning teams. Work with other students to study and discuss biology topics in lecture, as well as share your ideas and research predictions in lab. Teams are better learning environments but also, they are REAL LIFE. While scientists do some things on their own, they more often work in groups to solve problems because a well-functioning team is the most efficient way to work. Working in the same group in both laboratory and lecture will allow you to become more familiar with each other so you will feel comfortable enough to discuss your biology questions. Although it may be easier for an instructor to run a class or lab without group work, numerous research studies have shown that working in groups and discussing science with your peers can increase your learning ***considerably*** (although you must strive to be a "cooperative" group). By pooling your knowledge, members of your group will get "stuck" less often, and be able to progress far beyond what any individual in the group could do alone.

Week	Before Lab Meeting	During Laboratory Meeting Activities & Assignments DUE
1	View "*Strangers*" Film	Film discussion, Quiz, Honey Guide paper
2	View "IDEO" Film	Film Quiz & Debrief, Writing INTROS, Form Groups
3	View "Islands" Film	Film Quiz, 4-slide Proposal Talk & movie, Grading TITLES
4	Group Contract	***2¶-Draft due***, Preparing for LA and Prof Thesis Interviews: Q&A
5	*GEA1* on Catme.org	**LA Interviews begin** (during & outside lab time, groups of 4, 60m)
6		LA Interviews (cont.) Writing RESULTS & FIGURES
7		***Half-Draft due*** (2¶+ Results/Figs paper), Grading FIGURES
8	*GEA2* on Catme.org	**PCR** & **Prof Interviews begin** (during lab, in groups of 2, 60min)
9-12		Gene research (PCR, gels, Primers, BLAST =Molecular Teams)
13		***Final film*** and/or ***Final paper (full DRAFT1) due***
14-15	*GEA3* on Catme.org	**Prof Interviews completed** (during lab, in groups of 2, 60min)

THE LABORATORY

You will need the Laboratory Manual resources provided in the Course Pack. Regularly, each week, revisit and review the lab guide materials provided to you in the Course Pack. This semester, you will design and pursue one experiment all semester long. You will find an interesting terrestrial animal behavior related to communication that has been studied and published in the literature (like a mating display) and attempt to document it when observing animals on campus (like squirrels & humans). 4.0-seeking students will also connect the behavior to a gene. Your group will capture your observations with still photographs and digital video from your smartphones. Ultimately, you may generate a short 5-minute documentary film showing the results of your research and write a formal research manuscript. Each week, you will examine and practice the methods of a scientist in performing your research. This approach is aimed at mentoring you, and providing regular practice, so you will master the ability to think and work like a serious scientist.

Participation and collaboration: While working on group projects, you should be mindful of other students in your group; therefore, it is important for all participants to exercise:

- Respect for themselves, each other
- Openness and a positive attitude toward new ideas and other's ideas
- Flexibility and tolerance of ambiguity
- Good communications amongst themselves
- *You, individually*, do observations every week, out in the field, and record it in your notebook
- *You, individually*, find new papers for your group's project, each week, and keep in a notebook
- Share your weekly data in your notebook and new papers you find, with your group and LAs

ASSIGNMENT SCHEDULE & VALUES

Speaking (value)	*Writing (value)*	*Discussing/Demonstrating*
Proposal talk & movie= 10%	Proposal 2P¶-Paper= 10%	LA Thesis interview= 10
Progress talk & movie= (ReDo)	Half-Draft Paper= 20%	Prof Thesis interview= 20%
Documentary movie= (e.c.)	Final Paper/Film=30%	Attendance & Participation= 10%

Week	**Assignment(s) Due**	**Value (%)**
(*all*)	Attendance & Participation	**10 (+ec)**
3	**Proposal Talk & movie**	**10**
4	First "**2 paragraph" ¶-*Paper***	**10**
5	*LA Thesis Interview* (individual score, group format)	**10**
7	Second "**Half-Draft" *Paper***	**20**
7-15	*Prof Thesis Interview* (individual score, pair format)	**20**
12	Final **"Draft1" *Paper*** and Film e.c. option	**20**

Total = 100% of lab grade

The "Honors Option" (optional)

*Note: The Honors Option for LB144 this semester is presenting your group's research findings as a poster or talk at the UURAF during the Spring Semester. This must be an individual assignment (not done as a group) and at an in-person setting (not virtual) if you seek individual credit for an Honors Option. Fyi: UURAF deadline is usually in January.

MSU & LBC INFORMATION AND POLICIES

Absence due to illness: Students who need to quarantine themselves, have been sick with COVID-19 symptoms, tested positive for COVID-19, or have been potentially exposed to someone with COVID-19 must follow CDC guidance to self-isolate or stay home. Illness or self-isolation will not harm performance or put one at a disadvantage in the class.

Technical Assistance

If you need technical assistance at any time during the course or to report a problem you can:

- Visit the Distance Learning Services Support Site
- Visit the Desire2Learn Help Site (http://help.d2l.msu.edu/)
- Or call Distance Learning Services: (800) 500-1554 or (517) 355-2345

Mental Health Resources

College students often experience issues that may interfere with academic success such as academic stress, If you or a friend is struggling, we strongly encourage you to seek support. Helpful, effective resources are available on campus, and most are free of charge.

- Drop by Counseling & Psychiatric Services (CAPS) main location (3rd floor of Olin Health Center) for a same-day mental health screening.
- Visit https://caps.msu.edu for online health assessments, hours, and additional CAPS services.
- Call CAPS at **(517) 355-8270** any time, day or night.
- 24-Hour MSU Sexual Assault Crisis Line **(517) 372-6666** or visit https://centerforsurvivors.msu.edu/

Resource Persons with Disabilities (RCPD)

- To make an appointment with a specialist, contact: (517) 353-9642
  Or TTY: (517) 355-1293
- Web site for RCPD: http://MYProfile.rcpd.msu.edu

Inform Your Instructor of Any Accommodations Needed

- From the Resource Center for Persons with Disabilities (RCPD): Once your eligibility for an accommodation has been determined, you will be issued a Verified Individual Services Accommodation ("VISA") form. Please present this form to me at the start of the term and/or two weeks prior to the accommodation date (test, project, etc.).

LBC Student Success and Advising Team

LBC advisors work to educate, coach, and support students in our college. For more information about the Student Success and Advising team visit: https://lbc.msu.edu/advising/index1.html

To make a zoom or phone appointment with an advisor visit: https://lbc.msu.edu/advising/advising-appointments.html

To review LBC Academic Policies, including LBC's Academic Grievance Policy, visit: https://lbc.msu.edu/advising/academic-policies.html

Related Policies:

Institutional Data Policy:
https://tech.msu.edu/about/guidelines-policies/msu-institutional-data-policy/
Student Privacy Guidelines and Notification of Rights under FERPA
https://reg.msu.edu/ROInfo/Notices/PrivacyGuidelines.aspx

Commitment to Integrity: Academic Honesty

Article 2.3.3 of the Academic Freedom Report states that "The student shares with the faculty the responsibility for maintaining the integrity of scholarship, grades, and professional standards." In addition, the (insert name of unit offering course) adheres to the policies on academic honesty as specified in General Student Regulations 1.0, Protection of Scholarship and Grades; the all-University Policy on Integrity of Scholarship and Grades; and Ordinance 17.00, Examinations. (See Spartan Life: Student Handbook and Resource Guide and/or the MSU Web site: www.msu.edu.)

Therefore, unless authorized by your instructor, you are expected to complete all course assignments, including homework, lab work, quizzes, tests and exams, without assistance from any source. You are expected to develop original work for this course; therefore, you may not submit course work you completed for another course to satisfy the requirements for this course. Also, you are not authorized to use answers provided by Chegg.com or CourseHero.com or similar "cheat" web sites, or use Artifical Intelligence (AI) agents like ChatGPT to complete any course work in this course. Students who violate MSU academic integrity rules may receive a penalty grade, including a failing grade on the assignment or in the course. Contact your instructor if you are unsure about the appropriateness of your course work. (See also the Academic Integrity webpage.)

LINKS TO UNIVERSITY POLICIES

- **Spartan Code of Honor**
- **Academic Integrity**
- **RCPD Disability Accommodations Statement**
- **Mental Health**
- **Tolerance and civility**
- **Religious Observance Policy**
- **Student Athletes**
- **MSU Final Exam Policy**

Owner's Manual
(With lots of ideas and text stolen from great authors, Drs. Alice Dreger and Tanya Noel)

Why is this an "owner's manual" instead of a syllabus?

Most syllabi contain only class schedule information. By contrast, this is more like an "owner's manual" like the sort that comes with a new car. If you read and use this manual, you will understand how this course works, and you will be able to keep the course running smoothly and do the regular maintenance required to avoid breakdowns. Of course, this course isn't a car. It's more like a bus tour. I believe that a university course is in its essence not a number, and not a topic, but a group of people who share a common goal of learning about some particular thing. In this sense, a course is like a bus tour, a tour to a place that is unfamiliar to most of us. As the teacher, I am the bus driver and chief tour guide. Each member of the course starts off at "home" intellectually and emotionally and comes to the bus station which is the classroom. We agree to "take the tour" together, to get on the bus and travel together for the length of the course even though many of us may never have met before. Together we visit a number of different "places."

So why is this "owner's manual" so long?

I've discovered that the more information I give students, the more comfortable and in control they feel, and the better they learn. This packet contains lots of information. Besides telling you about the mechanics of the course, this packet tells you a lot about my teaching style. I used to provide my students with a separate "statement of teaching philosophy." It now occurs to me it is weird to separate that teaching philosophy from my teaching materials. So now my philosophy is embedded throughout this packet. My teaching style, methods, and philosophy change over time, thanks to students who tell me what works and what doesn't work. I'm counting on you to give me lots of feedback about what is working for you and what is not, and most importantly why. It is very important to me to do a good job for you. In addition to the course learning objectives provided earlier, be aware this course aligns with the following MSU Undergraduate Learning Goals:

Analytical Thinking
A successful student uses ways of knowing from mathematics, natural sciences, social sciences, humanities, and arts to access information and critically analyzes complex material in order to evaluate evidence, construct reasoned arguments, and communicate inferences and conclusions.

- *Acquires, analyzes, and evaluates information from multiple sources.*
- *Synthesizes and applies the information within and across disciplines.*
- *Identifies and applies, as appropriate, quantitative methods for defining and responding to problems.*
- *Identifies the credibility, use and misuse of scientific, humanistic and artistic methods.*

Effective Communication
A successful student uses a variety of media to communicate effectively with diverse audiences.

- *Identifies how contexts affect communication strategies and practices.*
- *Engages in effective communication practices in a variety of situations and with a variety of media.*

Integrated Reasoning
A successful student integrates discipline-based knowledge to make informed decisions that reflect humane social, ethical, and aesthetic values.

- *Critically applies liberal arts knowledge in disciplinary contexts and disciplinary knowledge in liberal arts contexts.*
- *Uses a variety of inquiry strategies incorporating multiple views to make value judgments, solve problems, answer questions, and generate new understandings.*

How does this course work in terms of the day-to-day?

When we meet in-person for the lecture class, our meetings will consist of discussions of the readings and activities related to the topics we are investigating. You should complete the reading assigned for the day before you come to class, and spend enough time thinking about the readings before class. You should come to class ready to summarize the readings and to ask and answer questions about them. Homework and quizzes will often be given on the readings.

Always give yourself plenty of time to do your work, and feel free to contact me whenever you need help or clarification. I like teaching and not only do I feel good when you learn, often when you

learn something new, I learn, too.

Generally, we will stick very closely to the attached schedule, however, the point of this class is for you to learn, so if we need to change our scheduled plans to achieve that goal, we will do so. If you feel that you need things to be done somewhat differently in class in order for you to learn better, please let me know and I will work to adjust our schedule or classroom dynamics so that we can maximize learning.

So what's my feeling about teaching?

I love it! And I think it shows – my students have voted me "honorary member of the graduating class of Lyman Briggs" ("teacher of the year") about five times in the last fifteen years, I was given the Teacher-Scholar Award of MSU, the MSU Alumni Club of Mid-Michigan Quality in Undergraduate Teaching Award (nominated by MSU faculty and alumni for teaching) and and most recently the Outstanding Faculty Award by the ASMSU Senior Class Council (nominated by MSU graduating seniors for teaching). If you hear that I am tough, I am, but that's because I care about your learning. If I didn't care about your learning, I would have stayed at Stanford University.

I am delighted to have recruited amazing LAs to help you do well in the course. You will find that our LAs share my love of teaching, of biology, and dedication to helping you learn. But they are tough too because they want you to learn, lots. They are trained to answer your questions with responses in the form of guiding questions. Why? because it helps you learn and *remember*, and they know your next class (and career) will be far more difficult and demanding than this course, you know this too.

What else besides being in class will be required of you?

Note that this course uses a wider range of assignments than just several exams. This spreads out risk and stress so it's lower level, day to day, and allows you to assess your own learning with lower-stake quizzes to avoid any surprises when facing the bigger exams. Grades are pretty simple, like getting an "A" or "B+" or "C" written at the top of each assignment– and you can always check your grade on the D2L grade book – but be sure to keep your own spreadsheet and alert me if my grade sheet has an error.

- *Quizzes on readings:* I will frequently give short quizzes on a day's assigned reading at the beginning of the class meeting. These quizzes accomplish two things: (1) reward you for keeping up to date on the readings; (2) reward you for spending enough time on the readings to really understand them. If you read carefully, you should have little problem with the quizzes. If you have a lot of trouble with short, fast quizzes, remember there are lots of extra options in this class you can use as substitutions. If you miss a quiz because you are late or absent, you will receive a "0". These cannot be made up.

A note on grades & FERPA:

To support blind grading, we will often request that you not list your actual name but just provide your PID. Privacy, as required by MSU FERPA regulation, will be maintained by utilizing a code that is NOT your real MSU ID, so we'll call it your B-PID. Your B-PID will be listed on D2L in your personal grade book.

Backstory: In recent years universities have become very afraid of getting in trouble for breaking the law called FERPA (Family Educational Rights and Privacy Act). The law was created back in 1974 to protect the privacy of students and their grades. In response to it all universities created student ID numbers so instead of placing a grade next to a person's name, instructors could place it next to a student number to maintain privacy. Many universities chose to use a student's social security number to also be their student number. When identity theft became a big problem, universities then changed all their

student ID numbers from social security to become some number randomly generated in house. In recent years now the randomly generated student ID number itself has become protected. In fact, while other people are permitted to know your name, and even say it aloud and post it publicly, the student ID number is super protected. Thus, instead of using your officially MSU-issued ID, in this course, MSU requires that we issue a new temporary student ID. We will call these the B-PID, it's for blind grading.

Professors can use grades in two ways: they can use grades to "sort" students into "A" students, "B" students, etc.; or they can use grades as learning incentives and rewards. Unfortunately, the sorting system generally sorts according to "talents" students either have or don't have before they ever reach a particular classroom, e.g., the talent of being able to memorize and recall a lot of things. I would rather use grades to encourage students to develop their skills, to expand their minds and interests. While students are often only familiar with positive curving (sometimes called a mother's curve) a number of university classes use an actual curve that raises or lowers the grading scale with the goal to only permit a few students (like just 10 in a class of 100) to earn a 4.0 and then only a few (perhaps 20) are permitted to have a 3.5 etc. Even if everyone in the class got above a 90% on an exam, the grade scale would shift up until only the prescribed number of students got a 4.0 grade. This is a real "curve," and, I will never grade on a curve like this. Our grading scale with stay exactly as stated in the syllabus and each student will get whatever grade she or he has earned by the end of the semester. Nothing would make me happier than if everyone worked hard and learned a lot and got 4.0s. I would feel that we had achieved something great.

Table 1- University-level grading system: The table below describes the relationships between grades, percent, and performance in the University-level grading system used in our lab and lecture courses. The first column describes the letter/number grade. The second column describes the percentage associated with that grade. The third column describes the performance-level required. Remember, if at any point you feel confused or distressed about your grades, carefully review the syllabus, and talk to me.

Letter Grade	***Percentage***	***Performance***
A (4.0)	90 to 100%	*Outstanding Work*- A "4.0" is Outstanding. It literally stands out. It has the characteristics described for 3.0 and 3.5-level elements but in addition, the work by itself impressed with how much & well it was done. The student taught Prof something original.
B+ (3.5)	85 to 89.9%	*Most Excellent Work* - A "3.5" is Most Excellent. Every detail of the work was done extremely well, and they found additional papers and evidence beyond what they were told.
B (3.0)	80 to 84.9%	*Excellent Work* - A "3.0" score is considered Excellent. It is impressive work, top of the class, and the work was done extremely well but nothing beyond what was expected.
C+ (2.5)	75 to 79.9%	*Pretty Good Work*- A "2.5" is Pretty Good, the student did the minimum work required and did a pretty good job, this is expected at the university level and near average for the class.
C (2.0)	70 to 74.9%	*Average Work* - A "2.0" is average, the student did the minimum work required.
D+ (1.5)	65 to 69.9%	*Below Average Work* - the student did less than minimum work required.
D (1.0)	60 to 64.9%	*Poor Work*- the student did less than minimum work required and of poor quality.
F (0.0)	0 to 59.9%	*Failing Work*- the student did far less than minimum work required and very poor quality.

Course Structure

This course will use a public website and online tools like Turnitin, CATME, Desire2Learn, and Top Hat. The course website may include online lessons, course materials, and additional resources. Activities may consist of readings, discussion forums, email, journaling, wikis, and other online activities. You will need your MSU NetID to log in to the course to access the grades on *D2L* (*http://d2l.msu.edu*).

Definitions, terms, transparency

Admission: I believe caffeine and sugar increase attention and learning but have no empirical data to support this, except for eating donuts, which is documented to work, but just for 15 minutes post-eating. I like the drink called the Cortado (it's coffee, like a tiny latte) but particularly enjoy the moment I pour cane sugar out of the brown paper packet on top of the frothed milk and watch it sink into the drink. When you come to office hours, unless there's a rush, I'll likely offer you an espresso.

Attendance: Student learning is impacted by many things, yet education research has robustly shown it is significantly impacted by these three things: class size, teacher quality, and attendance. You are, of course, permitted to skip classes but often attendance is taken verbally in lab, and in lecture a single clicker point is made available to you, to encourage attendance since it correlates with learning. Attendance at the meeting of a class will be defined as being physically present in the room for the full time period of the class meeting. Thus, be present, in your seat with your notebook open and pen in hand, at the very beginning when the clock in the room strikes the hour and class begins, still there during/throughout the entire duration of the class, as well as at the very end of the official time period (feel free to come and go to visit the restroom, just not off vacationing elsewhere). It's only fair to treat students who arrive late exactly the same as those who depart early. We will often reward students for attendance by using technology to record their presence. If you fail at using your device to click in for attendance at the beginning middle or end of class, due to whatever reason, be aware we do not micromanage the attendance data (no appeals). If it happens, making the choice to schedule another course that has a start or finish time that is proximal or even overlaps with this class is, of course, your choice and entirely acceptable. Yet this will not change the definition of attendance or waive it. University students are adults and literally everything in a course is optional, yet if you want credit for attendance (and more importantly to learn) you must be there.

Belong: Lyman Briggs College is dedicated to promoting inclusion and fostering diversity. Let's make our classroom comfortable and welcoming for everybody. Let's strive to treat everyone with respect, civility, and empathy and rather than avoid new things to learn from others about different beliefs, practices, and lives. You are all super wonderful smart people, and all belong here.

Blind grading: When a computer scores a scantron bubble sheet from a multiple-choice exam, it is objective, it doesn't have a pre-conception as to which students are smart, or are nice to it, so it treats everyone the same and just rewards correct answers. Unfortunately, human graders are less objective. LAs, GTAs, and Profs, are all unable to be perfectly objective when they have already had interactions with the person whose work they are grading. While they try hard to be so, education research shows that even knowing what the person's name is will impact the grader and grade (even if they never met the person). Thus, imagine if they know the person reasonably well. If they have read prior papers, knew the person's prior grades, or had several positive (or negative) conversations with them. Wow, that will cause major problems when trying to be objective while grading, even for the best teacher ever, unless the grader is blind to the identity of the author. Professional journals and grant review panels use single blind or double-blind systems to avoid subjective evaluation. We will use this in our class too.

Participation: It turns out participation is different from attendance. It refers to a student who is actively working to perform the work and learn the materials discussed in the course. Students who are active participants do not merely talk during class but also prepare in advance for the class and do work outside of class. For the lecture course, this means carefully completing the readings, taking notes on them (best for learning, is to do this by handwritten notes on paper), and preparing for the upcoming class meeting by reviewing notes and highlighting any questions you thought of while preparing for class. To reward this behavior, which enhances learning, often there will be a pop quiz or problem or writing exercise during class which is scored. Also, there are clicker questions during the lecture, and you earn a point each time you choose a correct answer. There are also online lectures hosted on TopHat.com with readings and questions you can gain points for getting correct. You only need to get above 70% of all TopHat points to earn an Outstanding (4.0) grade for lecture participation. For the lab course, this means going out in the field, collecting data for your project each week, and recording it in your official lab notebook (trifecta style). It also means working well with your group, working just as much as your peers, and CATME as well as instructors' observations of you and your notebook, will be used to evaluate your lab participation. If you prepare well for class (lecture & lab courses), you'll get good grades, and if you don't, you are accountable. This helps increase the number of people who ultimately decide they need to study the material or collect data before class and as a result, also learn more when discussing the material again in class. If you prepare, the class is fun and interesting. If you don't, it can become confusing and frustrating, as it feels like everyone else seems to know all the answers while you don't even understand the questions. The lab participation grade represents half of the final combined Attendance and Participation grade. Here are examples of Outstanding versus 0% participation in the lab course. OUTSTANDING participation would be: you did all CATMEs, got high scores on all, have many full experiments listed in your lab notebook, and many highlighted/read papers on your gene, animal, and behavior kept in a notebook. A 0% participation would be: you did no CATMEs, got low scores on all, have zero full experiments listed in your lab notebook, and found/read/contributed zero papers on your project's gene or animals or behaviors.

Random calling in the lecture: How often have you been in a big lecture class that has maybe 8 students who are the only people who ever are called upon to answer the professor's questions in the lecture? The other 100+ students throughout the entire semester will generally never speak aloud during lectures. After a while, you get used to it. Everyone knows that "those students" answer the questions, so we don't have to, cool. Yet deep down you also know, that while it's comfortable to never have to answer a question, it likely reduces your learning, heck some folks fall asleep. My wife tells a story about a small class where the Professor always asked these incredibly difficult questions that nobody ever even understood. Then one day, near the end of the semester, for the very first time, she did the reading before class and during class realized that every single question the instructor asked had always been directly out of the reading. She was embarrassed because she realized the Professor must know nobody does the reading, given no student ever understood the questions he asked, even though they were right out of the first pages of each reading. Because our goal in this class is learning we will use random calling in lectures to help *everyone* increase their learning and gain skills in communication/public speaking. Given that our #1 goal in the course is you to become comfortable and confident at public speaking, this is required to help that happen.

What is inheritance? This is when the grade of a second assignment, e.g. a second paper, a second talk, or a second exam might replace the grade of the first paper, talk, exam, etc. to reward improvement.

What are ReDos? This is an opportunity to retake an exam or repeat an assignment and have the new score/grade replace the old one (even if it is lower). This is a second chance at learning.

What are BUMP points? In the lab course, these are percentage points awarded to students each week, which elevate the final score of their next Exam. They are earned by those who demonstrate their high-quality work by showing instructors their full experimental records in their official lab notebooks as well as new research papers they found and read on their research project and explaining them.

TARDIS pass: If you win a TARDIS pass, through high attendance and participation, it can be used to ReDo an assignment. It is similar to the Formal Written Appeal Process outlined on page 2 of the syllabus. It can be used for everything except the Final Exam itself (due to time constraints). All discussion concerning score changes must be completed within 7 days from the date the grade was officially posted (on the returned assignment or online). So alert Luckie soon, within 7 days, if you wish to use your TARDIS pass to use time travel and ReDo an assignment.

Our no-points grading system: The grading system in the course is based upon the University Grading Scale (described earlier) and grade levels are described by terms e.g. "Pretty Good", "Excellent", "Outstanding" which are equivalent to 2.5, 3.0, 4.0. While some assignments like TopHat, or rubrics for the papers, still use points in evaluation, their final overall grade becomes a grade-level not a precise point total. We actually do not track a point total as the grading system, nor do the instructors track you current grade in either the lecture course or lab course. We just work to make sure the data, the individual grades, are provided and accurate on the D2L grades page. In a University level course students are not treated like children, they have incredible math skills and are expected to do the mathematics needed to regularly calculate and track their own grades. Each assignment's grade often becomes a grade-level and in the end every assignment is just worth a portion (percentage) of the final grade.

Here are descriptions associated with each grade level.

PRETTY GOOD= If a student did mostly what was asked (nearly or at the very minimum required) AND a pretty good job of it, the grade awarded is a "Pretty Good" grade level.
EXCELLENT= If a student did everything that was asked (the very minimum required, but nothing beyond) AND did an excellent job in the work, the grade awarded is an "Excellent" grade level.
MOST EXCELLENT= If a student did everything required AND MORE (did the very minimum in all categories as well as more than the minimum in one or more) AND an excellent job.
OUTSTANDING= If the student did everything that was asked for AND went FAR above and beyond what was asked (more than the minimum in multiple categories) AND did an amazing job!

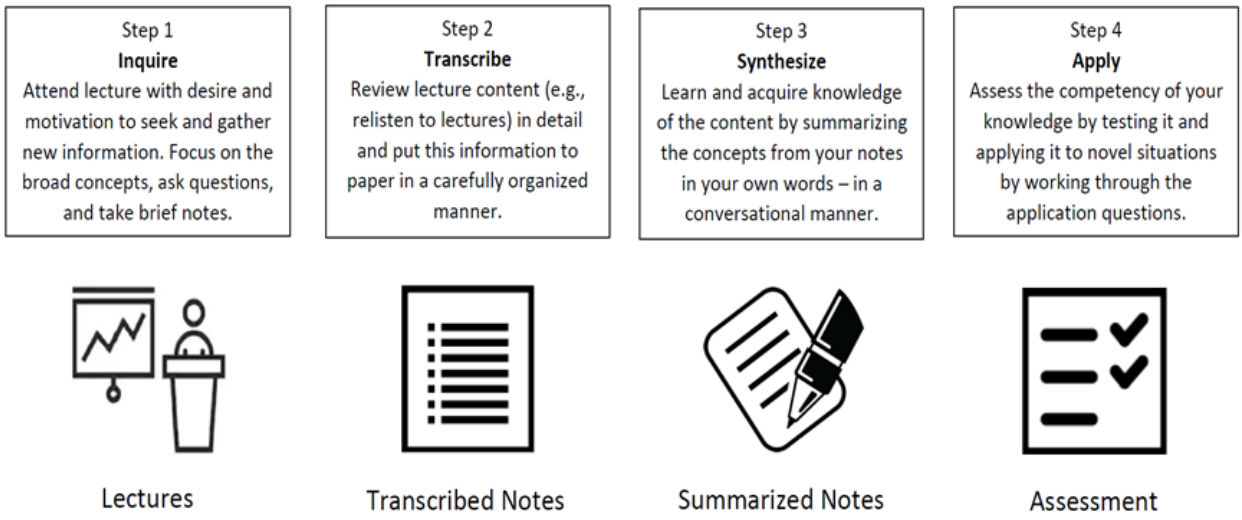
WHEN YOU NEED MORE LEVELS:
If a student did the minimum work required but not pretty good quality, just average quality =AVERAGE
If a student does less than the minimum work required =BELOW AVERAGE
If a student does less than the minimum work required AND of poor quality =POOR
If a student does FAR less than the minimum work required AND of poor quality= FAILING

NOTE: If the listed score for an assignment is not a descriptive "grade level" like those above, but instead just a number, e.g. 100% or 78.2%, which is always the case for exams, that is the final grade. It will not change to become a grade level.

At the end of the semester, in final grades calculations we will convert these grade levels to these numbers and do math to determine each student's final percentage grade: Outstanding (4.0 level) =95%, Most Excellent (3.5 level) =87.5%, Excellent (3.0 level) =82.5%, Pretty Good (2.5 level) =77.5%, Average (2.0 level) =72.5%, Below Average (1.5 level) =67.5%, Failing (1.0 level) =62.5%

Study Skills Tips from Dr. Marty Spranger (plus 18min video)

To gain deep understanding and be able to recall material try these four steps. Here is a link to an 18 minute video where he explains these steps. **Study skills for better learning**



Some Tips for students about technology use in the classroom from Dr. Tanya Noel

Almost everyone has a smartphone, laptop, tablet, or combination of these devices with them during their waking hours (and beyond, in some cases). There is huge potential for distraction using these– which is fine if you're waiting in a long, boring line or on a bus, but can be problematic in a class. Be aware of:

- There have been studies that have shown **"multi-tasking" in class is detrimental to learning**. (Actually, the evidence overwhelmingly suggests humans can't really multi-task … or, at least, can't multi-task well!) If you're trying to go back and forth between course-related stuff and other websites (or assignments for other courses, etc.), this will affect how well you're learning/working.

- **Notifications (e.g., beeps/vibrations for new emails, text messages, etc.) are highly distracting**, and feed into "reward systems" in the brain that can reinforce behaviors like frequently checking your phone, Facebook, etc. (You know that uncomfortable feeling that makes you check your phone/email? Your brain gets a dopamine hit when you give in to that urge … and makes it more likely to continue the behavior leading to the reward.) **Consider turning off these notifications**, at least during class and other times when you want to be able to focus uninterrupted. (Some people have found turning off notifications altogether has helped them not only focus but reduced their stress levels!)

- **Note-taking on computers is associated with lower-quality learning/test scores (vs. by hand)**. Results from some recent studies support the idea that writing notes by hand on paper is superior to taking notes on the computer. There are several hypotheses about this, but many experts agree that taking notes by hand involves more thinking about what's important and worth writing down (as you can't transcribe every word spoken by the professor). On the computer, it is tempting to try to record everything verbatim, with the brain not processing much of the information. Touch typing by definition is writing by reflex as a result of training, by not thinking at all really.

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Chapter Checklists for each lecture

I strongly suggest attaching each Checklist sheet for the current week to your "work wall" (e.g. bulletin board) in your room where you can see it at a glance and literally check-off items as you complete them.

Before first day of class:

Obtain supplies for course: online textbook, online homework system, and official course pack. *See course website for more details (http://ctools.msu.edu/144)

1. _____ **Buy** our online **Integrating Concepts in Biology (ICB) textbook**. Do not buy an expensive $259 Biology textbook! Please just buy this inexpensive one. Note: this textbook is custom-assembled with just the chapters for this class, so unless you want the full textbook, buy the LB144 Luckie version of the ICB textbook (http://store.trunity.com/ just search for "Luckie").

2. _____ **Buy** access to **TopHat online homework** & **clicker system.** Do not spend $100+ for an online homework system like Mastering Chemistry/Biology and then also buy a $50 clicker you might break or lose. Just buy the less expensive TopHat online homework system for the semester, which also permits you to use your phone/tablet/laptop as a clicker. Click the TopHat.com link on course website.

3. _____ **Buy** traditional paper **Lecture Notebook.** Can be spiral bound or 3-ring bound as long as there is real paper that you'll write on lots (mostly for taking notes when doing the readings prior to lecture). Be sure to write all notes by hand, it greatly increases your learning.[1]

4. _____ **Buy** the **LB144 Course Pack** (for students in Luckie's sections). Keep it somewhere you can easily access. We will use it quite a bit, particularly when in Lab Meetings. This Course Pack contains the syllabus, lecture handouts, learning objectives, lab manual, scientific papers and lab notebook. A pdf of the Course Packet will also always be available online for easy 24/7 access.

[1] **Mueller PA, Oppenheimer DM. 2014. The Pen Is Mightier Than the Keyboard: Advantages of Longhand Over Laptop Note Taking. Psychol. Sci. 25:1159–1168.** (https://www.npr.org/2016/04/17/474525392/attention-students-put-your-laptops-away)

Lecture1 - The LIVE in-person lecture (crickets call)

Budgeting homework time (50 min): Ch. 18, section 18.1 (the first half on **crickets**) is approximately 1775 words in length. At what's considered slow reading speed, 200 words per minute, reading the first half of section 18.1 should take 9 minutes. But when done properly, when you pause to review figures, read and think about a few of the Integrating Questions, and take careful notes, if you focus (avoid distraction) it should take you approx. 50 minutes.

1. _____ For the first lecture, read the 1-page **Foreword** at the beginning of the textbook written by the very famous Dr. Bruce Alberts, review the Student Resources in **Chapter 0**, and then begin reading **Chapter 18: Information in the Environment** of our book, Integrating Concepts in Biology (ICB). Read the single Introduction page. You do not need to take notes on any of these pages.

2. _____ Then slowly read the section we will discuss most during lecture, section 18.1 "Have organisms evolved to exploit communication between individuals of other species?" As you read section 18.1 (the part on crickets, not frogs) on your computer be sure to **take handwritten notes on paper in your lecture notebook**. Handwritten notes lead to much greater learning.

3. _____ **Try to answer at least one Integrating Question (IQ) in each set**. As you read the ICB textbook always attempt to answer at least one of the yellow **Integrating Questions** each time you get to a set of them. It will help you test yourself to determine if you got the meaning, or not, while reading the last few paragraphs. Just like taking handwritten notes, this too will greatly increase your learning. If you desire a high grade in the course, try to answer more IQs.
   NOTE: Assume you will be asked a question in lecture which is directly from one of the IQs.

4. _____ **Trifecta**: **Prepare to explain (aloud) Figures 18.2, 18.3, 18.4 and Table 18.1 in class**. As you read a section from the ICB textbook always attempt to pause and study **each figure/drawing/table** that is discussed. Some of them are just pictures or drawings and may not require lots of thinking, but others are graphs or tables that contain actual data from research experiments. Spend more time looking at these. In class, during lecture, students will be randomly chosen to explain a Figure or Table aloud (LA will hand you a microphone) so prepare for when your name is called to be sure you are ready. *Some students avoid stress by just writing out in their notebook an explanation of the Purpose, Methods and Findings of each data figure* (we call these three things *the Trifecta*). If it's already written down then you can just read aloud what you wrote, like: "Purpose: Dr. Griffith wanted to determine...., Methods: his group worked with mice and pneumonia bacteria called...., Findings: in the end they found evidence that ...".

5. _____ **Advanced TIP reported from prior student**: "The way the textbook explained this figure did not make sense to me, so I scrolled down to the bottom of the page and clicked on link to the original paper and read about the same figure in that. The way the paper explained it made so much more sense and cleared up what I was confused about. Tell other students about this!"

Lecture2 - (Preparing for) **This week's TOP HAT online lecture (frogs sing)**

Budgeting homework time (45 min): Ch. 18, section 18.1 (the second half on **frogs**) is 2166 words in length. At what's considered slow reading speed, 200 words per minute, reading the second half of section 18.1 should take 11 minutes. But when done properly, when you pause to review figures, read and think about a few of the Integrating Questions, and take careful notes, if you focus (avoid distraction) it should take you approx. 45 minutes.

1. _____ **For the second lecture,** slowly read the second half of section 18.1 "Frog choruses attract predators." As you read it on your computer or tablet, please be sure to **take handwritten notes on paper in your lecture notebook** (handwritten notes lead to far greater learning).

2. _____ **Try to answer some Integrating Question and Review Questions.** As you read the ICB textbook always attempt to answer at least one of the yellow **Integrating Questions** each time you get to a set of them. Also try to answer the green Review questions.

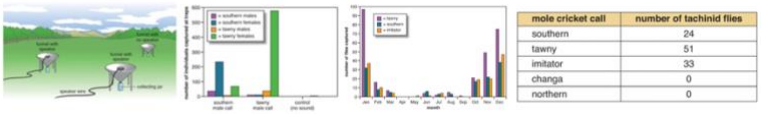
3. _____ (Trifecta): **Prepare to explain (aloud) Figures 18.6 and Table 18.2**. As you read a section from the ICB textbook always attempt to pause and study **each figure/drawing/table** that is discussed. In LIVE classes, during lecture, you will be chosen to explain these aloud, SO on TopHat days practice, practice, practice, even stop the video and say your Trifecta aloud for the Figure or Table being discussed. Thus, you will be super ready and confident.

4. _____ **Advanced TIP**: scroll down to the bottom of the page, in the Bibliography, and click on the link to an original paper by Dr. Rachel Ryan to see which data was used to make figure 18.6 and Table 18.2, and look at Ulagaraj's research paper on crickets to get used to, and in a habit of, checking original papers.

JClub1 - (Preparing for) **The LIVE in-person lecture (Ulagaraj + Page papers)**

Have organisms evolved to exploit communication between individuals of other species? (Prey Detection)

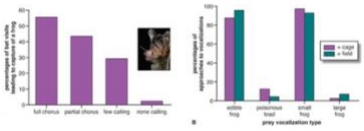
(18.1)



mole cricket call	number of tachinid flies
southern	24
tawny	51
imitator	33
changa	0
northern	0

Raj Ulagaraj, T. Walker: Phonotaxis of crickets in flight: attraction of male and female crickets to male songs, *Science* 182(4118):1278, 1973.

(18.1)



treatment	mean number of trials	standard error	sample size
inexperienced bat with experienced bat	5.3	1.7	10
two inexperienced bats	96.8	3.2	5
one inexperienced bat	96.2	3.8	5

Rachel Page, M. Ryan: Social transmission of novel foraging behavior in bats: frog calls and their referents, *Curr Biol* 16(12):1201-1205, 2006.

Lecture3 - (Preparing for) **The LIVE in-person lecture (corals settle)**

Budgeting homework time (60 min): In Ch. 18, section 18.3 (the second half of on **coral reefs)** is 1932 words in length which should take 10 minutes if you just read it. But when done properly, when you pause to review figures, read and think about a few of the Integrating Questions, and take careful notes, this homework assignment should take you more like 60 minutes (and that's if you are not distracted).

1. _____ **For the third lecture of the semester, read the** second half of section 18.3, after the blue header titled "Information is used by corals during settlement" and as you read it on your computer be sure to take handwritten notes*. You should focus and take detailed notes for everything about coral. Don't worry about the first part of 18.3 where the reading is about moths.

2. _____ **Try to answer some Integrating Question and Review Questions.** As you read the ICB textbook always attempt to answer at least one of the yellow **Integrating Questions** each time you get to a set of them. Also try to answer the green Review questions on coral reefs.

3. _____ (Trifecta): **Prepare to explain (aloud) Figures 18.18, 18.19, and 18.20 in class** (Purpose, Methods, Findings)

4. _____ **Advanced**: Click on the last reference in the Bibliography at the bottom of the page and try to find Figure 1 (not Plate 1) in Dr. Lindsay Harrington's research paper in the journal *Ecology* in 2004. Just take a peek, read the abstract and in her paper where they talk about Figure 1.

Lecture4 - (Preparing for) **This week's** TOP HAT **lecture (fireflies)**

Budgeting homework time (70 min): In Ch. 17, section 17.1 is 262 words, and section 17.2 (the first half on **fireflies**), is 2569 words in length, together totaling almost 2900 words. This should take 15-20 minutes if you just read it. But when done properly, when you pause to watch the three short movies, and then review a few data figures, read and think about a few of the Integrating Questions, and take careful notes, this assignment should take you 70 minutes (if you are focused).

1. _____ **For this lecture first read Chapter 17's,** very short, section 17.1.

2. _____Then slowly carefully read the first half of section 17.2 "How is information transmitted between members of animal species?" For section 17.2 you only need to carefully take handwritten notes in the section "Simple communication in a firefly". Be sure to watch the three short movies about the research of Dr. Sara Lewis.

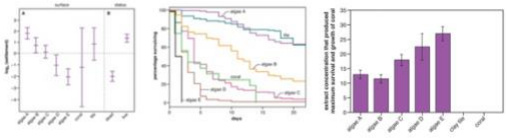
3. _____ **Try to answer some Integrating Question.** As you read the ICB textbook always attempt to test yourself a little, answer at least one of each set.

4. _____ (Trifecta): **Prepare to explain (aloud) Figures 17.3, 17.4, and 17.5**. Practice, practice, even pause the video and say your TopHat Trifecta aloud. Thus, you will be very ready and very confident when called upon to speak aloud in class.

JClub2 - (Preparing for) **The LIVE in-person lecture (Lewis + Harrington papers)**

Is chemical communication used to block competition or defend self? (Competition)

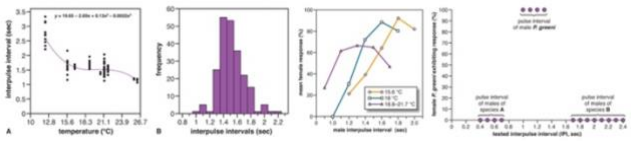
(18.3)



Lindsay Harrington, Katharina Fabricius, et al: Recognition and selection of settlement substrata in corals, *Ecology* 85(12):3428-3437, 2004.

How is information transmitted between members of animal species? (Populations, Communication, Behavior)

(17.2)



Sara Lewis, Michaelidis C, Demary K: Male courtship signals & female signal assessment in fireflies, *Behavioural Ecology* 17:329-35, 2006.

Lecture5 - (Preparing for) **The LIVE in-person lecture (storm petrels)**

Budgeting homework time (70 min): In Ch. 17, section 17.2 (the second half of on **birds**) is 2378 words in length. Technically this should take 12 minutes if you just read it. But when done properly, when you pause to review all of the data figures and tables, read and think about a few of the Integrating & Review Questions, and take careful notes, this assignment should take you more like 70 minutes (if you are focused). **Special Allowance**: Your group can divide up the Trifectas for this lecture.

1. _____ **For this lecture,** slowly read the second half of section 17.2 titled "More complex communication in a bird" (on storm petrels) and take handwritten notes.

2. _____ **Answer some Integrating Question and Review Questions.** As you read the ICB textbook always attempt to answer at least one of the yellow **Integrating Questions** each time you get to a set of them. Also try to answer the green Review questions.

3. _____ (Trifecta): **Prepare to explain (aloud) Figures 17.6, 17.7 and Tables 17.1, 17.2, 17.3 in class** (Purpose, Methods, Findings)

* **Special Allowance** today*: If you wish, your group can designate who will be responsible for each figure or table and thus reduce the load. YET realize you'll be expected to know YOUR figure/table very well, including the detailed Methods used, and what it said about the figure in the research paper too.

Lecture6 - (Preparing for) **This week's** TOP HAT **lecture (Meerkats)**

Budgeting homework time (50 min): In Ch. 17, section 17.3 (the first half on **meerkats**) is 1547 words in length. At 200 words/min this would take less than 10 minutes if you just quickly read it. But when done properly, when you pause to watch the two short movies, and then review several figures, read and think about a few of the Integrating Questions, and take careful notes, this assignment should take you 50 minutes (but longer if you are distracted by texts, friends, email etc).

1. _____ **For lecture, slowly** read the first half of section 17.3 on meerkats that asks the question: "Does group living require more derived mechanisms of information transfer?". You can stop reading when you finishing reviewing Integrating Questions 25-27. Please carefully take written notes on this reading in your lecture notebook.

2. _____ **Try to answer some Integrating Question.** As you read the ICB textbook always attempt to test yourself a little, answer at least one of each set.

3. _____ (Trifecta): **Prepare to explain (aloud) Figures 17.9, 17.10, and 17.11**. Practice, practice, even say your TopHat Trifecta's aloud at the appropriate time of the video. Thus, you will be very ready and very confident when called upon to speak aloud in class.

4. _____ **Advanced**: Read on further, about Dr. Martha Manser's research on meerkats and check the Bibliography to look at one of her papers.

(Preparing for) **Exam I and Marta Manser's papers (Meerkats continued).**

Budgeting homework time (50 min): In Ch. 17, section 17.3 (the second half on **meerkats**) is 1180 words in length. This should take 6 minutes if you just read it. But when done properly, when you pause to watch one short movie and then review three figures, read and think about a few of the Integrating Questions, and take careful notes, this assignment should take you 50 minutes (if you are focused).

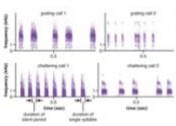
1. _____ **For lecture,** read and take handwritten notes on the second half of section 17.3 on meerkats (start after Integrating Question 27).

2. _____ (Trifecta): **Prepare to explain (aloud) Figures 17.12, 17.13, and 17.14 in class**.

3. _____ **Advanced**: Read on further, about Dr. Martha Manser's research on meerkats by looking at one of her papers in the Bibliography.

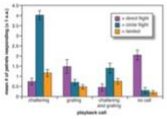
JClub3 - (Preparing for) **The LIVE in-person lecture (Bretagnolle and Manser papers)**

(17.2)



situation of the bird	call uttered		
	chattering	chattering and grating	grating
bird alone (*n* = 96)	38	35	23
flown over by another bird (*n* = 66)	33	15	18
facing another bird (*n* = 90)	9	10	70

compared situations	χ^2	*p*-value
alone vs. flown over	2.05	0.36
flown over vs. facing another	41.5	1×10^{-9}
alone vs. facing another	48.7	2×10^{-11}

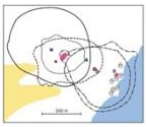


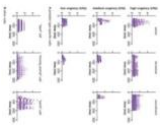
playback call	male response			
	chattering	chattering and grating	grating	no
chattering	0	0	15	8
male grating	0	0	12	7
female grating	0	0	0	1

Vincent Bretagnolle: Calls of Wilson's storm petrel: functions, sexual recognitions and geographic variation, *Behaviour* 111:98-112, 1989

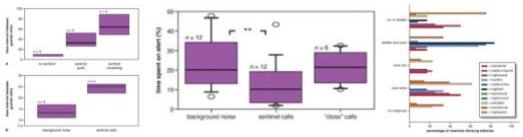
Does group living require more derived mechanisms of information transfer? (Social behavior, Eusociality)

(17.3)





Marta Manser: Response of foraging group members to sentinel calls in suricates, *Suricata suricatta*, *Proc Biol Sci* 266(1423):1013-1019, 1999.



Manser M, Bell M, Fletcher L: The information that receivers extract from alarm calls in suricates, *Proc Biol Sci* :268:2485, 2001.

Lecture7 - (Preparing for) **This week's** TOP HAT **lecture (Sandworts)**

Budgeting homework time (50 min): Read the second half of section 16.1 titled "Variation caused by the environment". This is just 1337 words with 3 figures. Just reading the text will take 8 minutes. Yet the figures contain data, thus, when done properly, when you pause to decipher each figure, try Integrating Questions, and take notes, this assignment will take you more like 50 minutes.

1. _____ **For lecture,** read the second half of section 16.1 titled "Variation caused by the environment".

2. _____ **Try to answer some Integrating Questions and Review Questions.** As you read the ICB textbook always attempt to test yourself a little, answer at least one of each set.

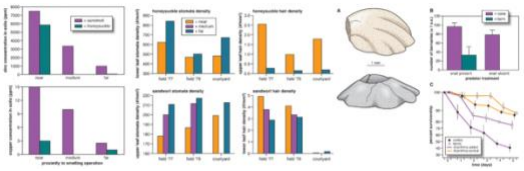
3. _____ (Trifecta): **Prepare to explain (practice, do it aloud, at the appropriate time during the videos) Figures 16.6, 16.7, and 16.8** (Purpose, Methods, Findings).

4. _____ **Advanced**: Use the papers in the Bibliography to learn more, particularly if you don't quite understand something in the figures. Find out where the figure came from and go see how the author explains the results. Maybe their explanation will make more sense to you.

JClub4 - (Preparing for) **The LIVE in-person lecture (Caiazza et al paper)**

What causes individual variation? (Variation and Population Genetics)

(16.1)



Nicholas Caiazza, Quinn JA (1980) Leaf morphology in A.patula & L.japonica along pollution gradient. Bulletin Torrey Bot. Club 107(1): 9-18.

Lecture8 - (Preparing for) **This week's** TOP HAT **lecture (Griffith):**

Budgeting homework time (60 min): Chapter 1, has a cover page, section 1.1 is 660 words, and section 1.2 on Drs. Griffith and Avery is 1725 words in length. While this is about 2300 words in total, thus the reading would be estimated to take 12 minutes. Yet careful reading and notetaking takes time and it has four data figures. While the Trifectas are easy to prepare for, Table 1.1 may be more difficult.

1. _____ **For lecture,** start **Chapter 1: Heritable Material** by reviewing the cover page and reading the short section 1.1: "What is biological information?" No notes needed.

2. _____ **Now slowly carefully read** section 1.2: "What is the heritable material?" and take careful handwritten notes in your lecture notebook.

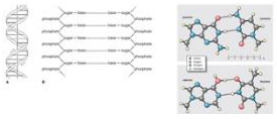
3. _____ **Try to answer some Integrating Questions** and **Review Questions**.

4. _____ (Trifecta): **Prepare to explain (practice, aloud) Figures 1.2, 1.3, 1.4 and Table 1.1** (Purpose, Methods, Findings).

JClub5 - (Preparing for) **The LIVE in-person lecture (Watson and Crick et al papers)**

What is biological Information? (Heritable Material)

(1.4)



Watson JD, Crick FH. nucleic acids. Wilkins MH, et al. Molecular ... Franklin RE, Gosling RG. Molecular configuration. Nature 171:737, 1953. (all four!)

Lecture9 - (Preparing for) **This week's** TOP HAT **lecture (Epigenetics)**

Budgeting homework time (45 min): Chapter 1, section 1.5 on Epigenetics is 1840 words in length. At 200 words per minute, reading section 1.5 should just take 10 minutes. But when done properly, when you pause to review figures, read and think about a few of the Integrating & Review Questions, and take careful notes, this homework assignment should take you more like 45 minutes (if you focus).

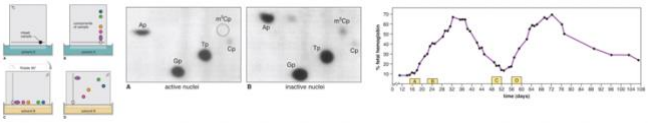
1. _____ **For lecture,** continue **Chapter 1: Heritable Material** by reading section 1.5: "Is all genetic information encoded linearly in the DNA sequence?" and take careful handwritten notes.

2. _____ **Try to answer some Integrating Questions** and **Review Questions**.

3. _____ **Prepare to explain (practice, aloud) Figures 1.19 (the method), and do a Trifecta for Figures 1.20, and 1.21** (Purpose, Methods, Findings).

JClub6 - (Preparing for) **The LIVE in-person lecture (DeSimone paper)**

(1.5)



DeSimone J, Heller P, Hall L, et al. 5-Azacytidine stimulates fetal hemoglobin synthesis in anemic baboons. PNAS 79(14):4428-4431, 1982.

Lecture10 - (Preparing for) **This week's** TOP HAT **lecture (Translation)**

Budgeting homework time (50 min): Chapter 2, section 2.3 is that is 1725 words in length with three data figures that require thinking and notetaking for the Trifecta. Reading at 200 words per minute would mean the section might take 10 minutes to read. Yet figures 2.20 and 2.21 are challenging and require time to think and read about them for the Trifecta. Of course, when done properly, when you pause to review figures, try Integrating Questions, and take notes, this assignment will take you more like 50 minutes.

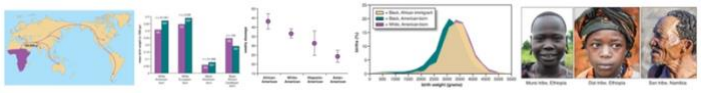
1. _____ **For lecture,** carefully read section 2.3: "How do cells make proteins?" and take handwritten notes in your lecture notebook.

2. _____ **Try to answer some Integrating Questions** and **Review Questions**.

3. _____ (Trifecta): **Prepare to explain (practice, aloud) Figures 2.20, 2.21 and 2.22** (Purpose, Methods, Findings).

JClub7 - (Preparing for) **The LIVE in-person lecture (Dang and Johnson papers)**

(6.5)



Dang MN, Hambleton J and Kayser SR. The Influence of Ethnicity on Warfarin Dosage Requirement. The Annals of Pharmacotherapy. Vol. 39: 1008 - 1012. 2005.

Johnson JA. Ethnic Differences in Cardiovascular Drug Response: Potential Contribution of Pharmacogenetics. Circulation. 118(13): 1383–1393. 2008.

Lecture11 - (Preparing for) **This week's** TOP HAT **lecture (Mendel)**

Budgeting homework time (60 min): Chapter 3, first 2/3's of section 3.1 is 2160 words in length with 7 figures that require thinking and notetaking. Reading at 200 words per minute would mean the section might take just 12 minutes to read. Of course, when done properly, when you pause to review figures, try Integrating Questions, and take notes, this assignment will take you more like 60 minutes. It could be shorter if you have been doing homework regularly, ie. training like an athlete, and getting stronger, better, faster at this now that it is week 7.

1. _____ **For lecture,** read section 3.1 on Gregor Mendel titled "How can traits disappear and reappear in a later generation?" Take careful notes by hand.

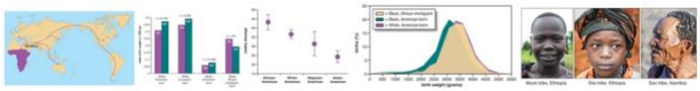
2. _____ **Try to answer some Integrating Questions and Review Questions.** As you read the ICB textbook always attempt to test yourself a little, answer at least one of each set.

3. _____ (Trifecta): **Prepare to explain (practice doing it aloud) Figures 3.3, 3.4, 3.6 and 3.7.**

4. _____ **Advanced**: Review how to use Punnett squares to predict the outcome of crosses.

JClub8 - (Preparing for) **The LIVE in-person lecture (Collins et al papers)**

(6.5)



Collins JW Jr, Wu SY, David RJ Differing intergenerational birth weights in Illinois. Am J Epidemiol. Feb 1;155(3):210-6. 2002

Lecture12 - (Preparing for) **This week's** TOP HAT **lecture (Evolution)**

Budgeting homework time (45 min): Ch. 4, section 4.1 is about 1250 words in length and ELSI 4.1 is 1100 words thus the total is 2350 words. At 200 words per minute, reading section 4.1 & ELSI should take 12 minutes. But when done properly, when you pause to review three figures, read and think about a few of the Integrating & Review Questions, and take careful notes, this homework assignment should take you more like 45 minutes (but longer if you are distracted by texts, friends, email etc.).

1. _____ **For the lecture, read** the cover page of **Chapter 4: Evolution and Origin of Cells** in the ICB textbook, but you do not need to take notes on that page. Then slowly read section 4.1 "What is evolution?" and as you read it on your computer be sure to take handwritten notes*. Last, read the section Ethical, Legal, Social Implications (ELSI) 4.1: "Are evolution and religion compatible?" You do not need to take notes on the ELSI reading, just think about it.

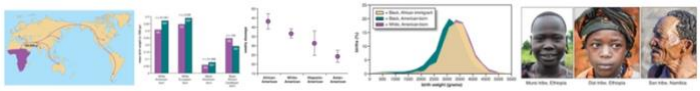
2. _____ **Try to answer Integrating Question and Review Questions.** As you read the ICB textbook always attempt to answer at least one of the yellow **Integrating Questions** each time you get to a set of them. Also answer the green Review questions.

3. _____ (Trifecta): **Prepare to explain (practice, aloud) Figures 4.1, 4.2 and ELSI Figure 4.1**. As you read a section from the ICB textbook always attempt to pause and study **each figure/drawing/table** that is discussed.

4. _____ **Advanced TIP**: click on the link to an original version of Darwin's Origin of Species, peek at it.

JClub9 - (Preparing for) **The LIVE in-person lecture (Ingman paper)**

(6.5)



Ingman M, Kaessmann H, Pääbo S, and Gyllensten U. Mitochondrial genome variation and the origin of modern humans. Nature. Vol. 408: 708 - 713. 2000.

Lecture13 - (Preparing for) **This week's** TOP HAT **lecture (Competition)**

Budgeting homework time (60 min): In Ch. 4, the first half of section 4.3 is 2000 words in length. This should take 10 minutes if you just read it. But when done properly, when you pause to review quite a few figures, read and think about a few of the Integrating Questions, and take careful notes, this homework assignment should take you more like 60 minutes (if you are focused).

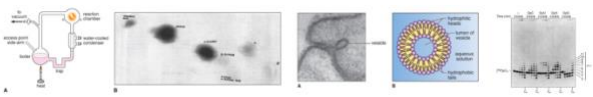
1. _____ **For lecture, read Chapter 4's** section 4.3(1st half) in the ICB textbook. For section 4.3 "Can non-living objects compete and grow?" you only need to carefully read and take notes on items up to and including information related to Figure 4.13. Be sure to take handwritten notes. Then stop taking notes and just read the rest to learn about research on vesicles competing with each other. Explore Bio-Math Exploration 4.2 if you find it interesting.

2. _____ **Try to answer some Integrating Question and Review Questions.** As you read the ICB textbook always attempt to test yourself a little, answer at least one of each set.

3. _____ (Trifecta): **Prepare to explain (practice, aloud) Figures 4.11, 4.12, and 4.13**.

JClub10 - (Preparing for) **The LIVE in-person lecture (Miller paper)**

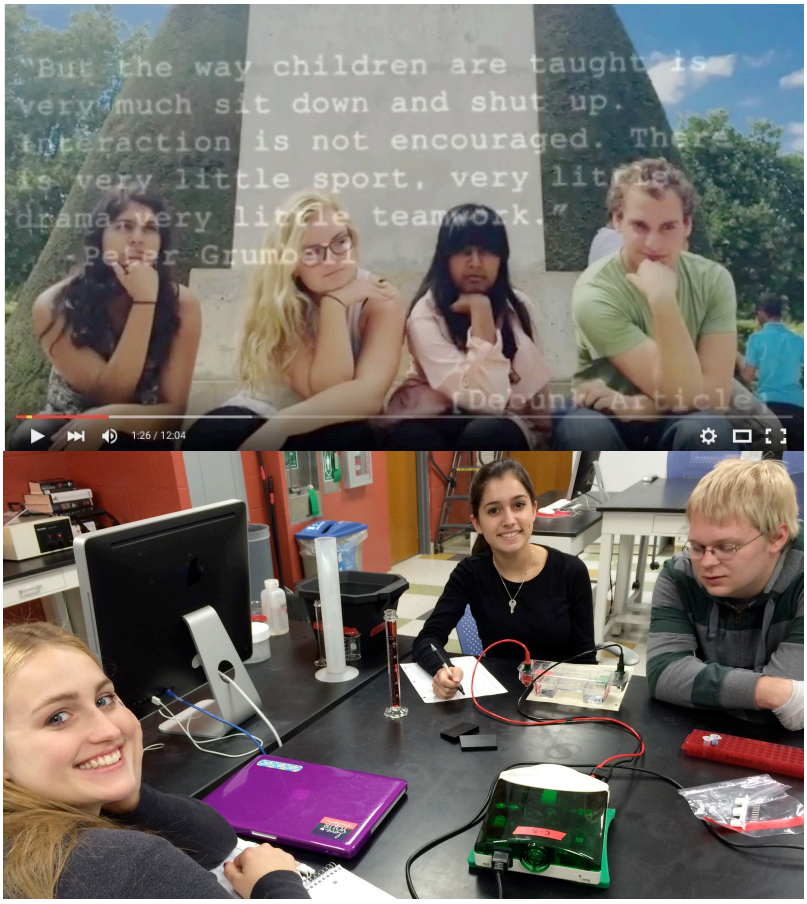
(4.2)



Miller SL. A production of amino acids under possible primitive earth conditions. Science 117(3046):528-529, 1953.

BIOLOGY I

Laboratory Guide



Phase I: Documenting animal communication and homologs in humans

&

Phase II: Seeking homologous genes for communication in animal and humans

Igert, Wright, Fata-Hartley, Haenisch, Cooper, Krha, Maleszewski, Wilterding, Sayed, Luckie

HYBRID EDITION

LB-144: CELL & ORGANISMAL BIOLOGY (LABORATORY)

(ALERT: *you must complete online safety training at ehs.msu.edu to work in the lab room*)

Lab will meet both on Monday mornings in E-26A and Wednesday afternoons in C-4 Holmes Hall

LAB COORDINATOR

Douglas B. Luckie, Ph.D., Professor, Lyman Briggs College & Department Physiology

LAB MANUAL

found inside "LB-144 Course Pack," (Luckie) from MSU Library Services via local bookstore

COURSE WEBSITE http://ctools.msu.edu/144

RESEARCH TEAM RATIONALE

Student groups are intended to be research & learning teams. Work with other students to study and discuss biology topics in lecture, as well as share your ideas and research predictions in lab. Teams are better learning environments but also, they are REAL LIFE. While scientists do some things on their own, they more often work in groups to solve problems because a well-functioning team is the most efficient way to work. Working in the same group in both laboratory and lecture will allow you to become more familiar with each other so you will feel comfortable enough to discuss your biology questions. Although it may be easier for an instructor to run a class or lab without group work, numerous research studies have shown that working in groups and discussing science with your peers can increase your learning ***considerably*** (although you must strive to be a "cooperative" group). By pooling your knowledge, members of your group will get "stuck" less often, and be able to progress far beyond what any individual in the group could do alone.

Week	Before Lab Meeting	During Laboratory Meeting Activities & Assignments DUE
1	View "*Strangers*" Film	Film discussion, Quiz, Honey Guide paper
2	View "IDEO" Film	Film Quiz & Debrief, Writing INTROS, Form Groups
3	View "Islands" Film	Film Quiz, 4-slide Proposal Talk & movie, Grading TITLES
4	Group Contract	***2¶-Draft due***, Preparing for LA and Prof Thesis Interviews: Q&A
5	*GEA1* on Catme.org	**LA Interviews begin** (during & outside lab time, groups of 4, 60m)
6		LA Interviews (cont.) Writing RESULTS & FIGURES
7		***Half-Draft due*** (2¶+ Results/Figs paper), Grading FIGURES
8	*GEA2* on Catme.org	**PCR** & **Prof Interviews begin** (during lab, in groups of 2, 60min)
9-12		Gene research (PCR, gels, Primers, BLAST =Molecular Teams)
13		***Final film*** and/or ***Final paper (full DRAFT1) due***
14-15	*GEA3* on Catme.org	**Prof Interviews completed** (during lab, in groups of 2, 60min)

THE LABORATORY

You will need the Laboratory Manual resources provided in the Course Pack. Regularly, each week, revisit and review the lab guide materials provided to you in the Course Pack. This semester, you will design and pursue one experiment all semester long. You will find an interesting terrestrial animal behavior related to communication that has been studied and published in the literature (like a mating display) and attempt to document it when observing animals on campus (like squirrels & humans). 4.0-seeking students will also connect the behavior to a gene. Your group will capture your observations with still photographs and digital video from your smartphones. Ultimately, you may generate a short 5-minute documentary film showing the results of your research and write a formal research manuscript. Each week, you will examine and practice the methods of a scientist in performing your research. This approach is aimed at mentoring you, and providing regular practice, so you will master the ability to think and work like a serious scientist.

Participation and collaboration: While working on group projects, you should be mindful of other students in your group; therefore, it is important for all participants to exercise:

- Respect for themselves, each other
- Openness and a positive attitude toward new ideas and other's ideas
- Flexibility and tolerance of ambiguity
- Good communications amongst themselves
- *You, individually*, do observations every week, out in the field, and record it in your notebook
- *You, individually*, find new papers for your group's project, each week, and keep in a notebook
- Share your weekly data in your notebook and new papers you find, with your group and LAs

ASSIGNMENT SCHEDULE & VALUES

Speaking (value)	*Writing (value)*	*Discussing/Demonstrating*
Proposal talk & movie= 10%	Proposal 2P¶-Paper= 10%	LA Thesis interview= 10
Progress talk & movie= (ReDo)	Half-Draft Paper= 20%	Prof Thesis interview= 20%
Documentary movie= (e.c.)	Final Paper/Film=30%	Attendance & Participation= 10%

Week	**Assignment(s) Due**	**Value (%)**
(*all*)	Attendance & Participation	**10 (+ec)**
3	**Proposal Talk & movie**	**10**
4	First "**2 paragraph" ¶-*Paper***	**10**
5	*LA Thesis Interview* (individual score, group format)	**10**
7	Second "**Half-Draft" *Paper***	**20**
7-15	*Prof Thesis Interview* (individual score, pair format)	**20**
12	Final **"Draft1" *Paper*** and Film e.c. option	**20**

Total = 100% of lab grade

The "Honors Option" (optional)

*Note: The Honors Option for LB144 this semester is presenting your group's research findings as a poster or talk at the UURAF during the Spring Semester. This must be an individual assignment (not done as a group) and at an in-person setting (not virtual) if you seek individual credit for an Honors Option. Fyi: UURAF deadline is usually in January.

Laboratory: Expectations & Effort

Time Commitment- "Don't be a tourist"

- Remember that the laboratory is an essential component of this class and is worth alot of points. **This means that you should be prepared to spend on average 3 hours inside the LB144 laboratory as well as 6+ hours outside of the lab with your group per week – so plan accordingly**.
- The above also means that if you explain to your research team that you are "busy" with social events every evening and intend to go home each weekend, realize you won't succeed in this course. *Do NOT be a TOURIST* - make college a priority in your life.

The LB144 laboratory is a *RESEARCH TEAM LAB*

- Your research group will need to meet twice weekly to do field research outside of class during the week as well as meet to coordinate the projects and papers you author as a group. It is essential that you read and prepare prior to coming to lab each week, because the experiments require a good amount of organization to complete in a timely manner.
- Please familiarize yourself with the concept behind this lab – it will help you to keep your "eyes on the prize" (so to speak) as the semester progresses.
- **Attendance WILL BE TAKEN** at the beginning of each lab, so it is important that you arrive ON TIME.
- The key to success in the laboratory class is to think and work like a scientist (avoid the mindset of a student simply doing the very minimum). Strive to work as though you are a professional scientist and this is your own research lab and project (which is true).

Group Roles

The research teams in LB-144 will usually consist of 4 individuals. You are expected to help out in all tasks but you will have one specialty (your primary job in the team). If your team consists of 3 individuals eliminate the "Primary Investigator" and divide those responsibilities among the team members. You will be documenting all of you biology research with audio/video. Your group creates: (1) an online research paper with figures that include graphs, photos, short clips of audio/video, and (2) a short (10m) documentary film that disseminates your research to increase public understanding of science.

-Primary Investigator *& Director* (PID) - *Plan!*

The primary investigator will be responsible for organizing meeting times, overall project planning, as well as troubleshooting throughout the investigation. It is your job to also be sure all members participate in discussions and you record notes. Ask questions when a member hasn't spoken, "What do you think, Jen?". In addition to sharing the final grade for each group paper, the PI is assigned and graded for writing specific sections of the paper as well as editorial duties on all sections. Film Project: 1º responsibility is leading the planning of the projects, storyboarding & editing of documentary film, aiding the direction of photography and audio capture.

-Protocol Expert *& Digital Editor* (PEDE) - *Protocol!*

This individual is responsible for overseeing the creation of scientific protocols for each week's independent investigation (written experiments and steps you plan to do). It is your job to worry about whether the protocol is appropriate and being followed exactly during the experiment. In addition to sharing the grade for each full draft paper, the PE is assigned and graded for writing sections of the paper as well as editorial duties on all sections in creating all complete papers. Film Project: 1º responsibility the digital editing (building) the documentary film and training others.

-Data Recorder *& Sound* (DRG) - *Record!*

The data recorded is responsible for recording and organizing the results and taking many pictures to document the team's efforts. It is your job to be sure everyone is keeping their notebook up to date and data is being recorded properly. In addition to sharing the grade for each full draft paper, the DRD is assigned and graded for writing sections of the paper as well as editorial duties on all sections in creating all complete papers. Film Project: 1º responsibility is sound recordings (audio) + digital editing of audio clips for online paper.

-Laboratory Tech *& Photography* (LTP) - *Hardware!*

This individual is responsible for learning the many experimental procedures and becoming an expert on how to use the various pieces of equipment. It is your job to read manuals and get the right equipment to work properly. In addition to sharing the grade for each full draft paper, the LT is as- signed and graded for writing certain sections of the paper as well as editorial duties on all sections in creating all complete papers. Film Project: 1º responsibility is video (photo/cinema) + editing of film clips for online paper.

Working Effectively in Small Groups

As you know, a great deal of the learning that occurs in our class is based upon your interactions with your peers in small groups. But working well in small groups does not happen by magic. There are things that I have to do as an instructor to make sure that you are getting the most out of your experience, such as identifying conflicts between group members that might hinder your work and having TAs listening closely to what you are saying as they stroll around the lab room so that we can help you get the most from your discussions or keep you on track.

You, too, have to work to make your groups all that they can be. Below, are some suggestions of effective small group work. Some of these will also clarify my expectations of you in your groups. Look this over carefully and raise questions in class or to me privately.

Individuals in Groups

There are two things that happen in small groups. Individual students share their thoughts, but those thoughts are also reacted to by one or more students. The group succeeds only insofar as each individual does actually share their thoughts; an individual who is not trying their best will diminish the group's experience. When I evaluate your group participation via GEA forms and TA feedback, I will take into account the quality of your group interactions- how well you did on your own in your group and how well the group did overall. It is up to each individual, and in particular the PI, to encourage teammates to work together to maximize your learning. If someone is not doing the reading or is not participating in lab enough, discuss the problem and see what you can do to help that person. You are all in your groups together!

Group Process

Groups are really processes, steps toward a goal. There are two types of goals toward which you are striving in our class. The first is growth as an individual. Group work should leave you feeling that you understand the material better than you could have had you worked only on your own. The second goal has to do with teamwork. Have you, and the group, learned something about group problem solving through your work together? Do you know how to better communicate with others from different backgrounds and who hold different perspectives? If so, you have achieved the second goal.

In order to make the group process work effectively, please follow these guidelines:

- Everyone should participate in your group.
- Everyone's contributions should be welcomed.
- Everyone should be heard. Listen to and hear one another; ask questions if you do not understand what a group member has said.
- No one should dominate group processes.
- A recorder should take notes for the group during a meeting. If your group meets more than once, the recorder role should be shared equally by all members through time.

Names: _____

_____

_____

_____

Section #: _____

Team Ground Rules Contract Form

Adapted from Dr. Deborah Allen, Univ. of Delaware and Dr. Rique Campa, MSU

Team Assignment

Purpose: To establish team *norms* in order to promote constructive & productive teamwork.

Directions: To work best groups require that all team members clearly understand their responsibilities to one another. These team ground rules describe the general responsibilities of every member to the team. You can adopt additional ground rules if your group believes they are needed. Your signature on this contract form signifies your commitment to adhere to these rules and expectations.

Some questions to discuss when thinking about these rules:

1) What are your professional goals (i.e., what would you like to do following graduation)? How will working in a team help you achieve some of your professional goals?
2) Besides class time, when are you available to work with your team members (exchange your class and work schedules)?
3) What is the best method(s) and time for your team members to contact you? Share the necessary phone number(s), e-mail addresses, etc... *NOTE:* This contact information is private, so should not be shared with others outside of your team, and should only be used for class-related communication.

All group members agree to:

1. Come to class and team meetings on time.
2. Come to class and team meetings with assignments and other necessary preparations correctly and thoughtfully completed.

Additional ground rules (add as many as you like; see examples on appendix of Smith (2007)):

Example: We will always meet on these two days each week at these times _____

If a member of the team repeatedly fails to meet these ground rules, other members of the group are expected to take the actions below. When filling in the *"If not resolved"* sections, think about how, when, and who will communicate dissatisfaction to offending team members. Reflect back on what you've learned thus far about your teammates in terms of what previous team experiences they have had, how you have worked together as a group thus far, and how each member tends to feel and deal with conflict.

Step 1: If not resolved, what will your team do? How? When?:

Step 2: Meet as a team with your lab instructor. If not resolved, what will your team do? How? When?:

Step 3: The quit or fire clause: If the steps above have been completed without resolving the problem, any team member may quit the team. Alternatively, if all other team members are in agreement, the offending team member may be fired from the team. In either case, the individual no longer working as part of a team is required to complete the remaining class activities and assignments individually.

The LB144 teaching team reserves the right to make final decisions to resolve difficulties that arise within a team. Before this becomes necessary, the team should try to find a fair and equitable solution to the problem.

Group Name:_____

Member's Names (printed), Signatures, and date:

1._____

2._____

3._____

4._____

Biology I Lab Project: Getting Started

Assignment: **Document a communication behavior in animals & then seek a homologous behavior in humans**

Build your research plan!

- [ ] 1. Find a few published research papers that document a novel behavior used by animals during communication (ideally about the same animal as you will study).

- [ ] 2. Propose a plan by which your group will document that behavior using observation and filming of your animals.

- [ ] 3. Also propose a plan to seek to detect and document that same/similar (homologous) behavior in humans.

- [ ] 4.0-level. Find a gene that may be connected to that same behavior (and might exist in both animals and humans)

- [ ] (?) Or come up with an even better idea, ie "Prof dude, we want to do something *insanely* cooler ...."

General Primers

(in this case the word *primer* means an introduction)

Primer 1: The Metric System

You will find it very hard to exist in lab this semester without knowing and being able to use the metric system. The short story is that a base unit (meters, grams, calories, joules, etc) is given a prefix to indicate the scale of the unit (kilo = 1000 base units; mili = 1 / 1000 base units). You should memorize at least the units in bold since they are the most likely to be used this semester and you should also be able to convert from one unit to another (ie: know how to convert 500mg into 0.5g).

List of metric prefixes, symbols, and their multipliers.

Prefix	Symbol	Numerical multiplier	exponential
yotta	Y	1,000,000,000,000,000,000,000,000	10^{24}
zetta	Z	1,000,000,000,000,000,000,000	10^{21}
exa	E	1,000,000,000,000,000,000	10^{18}
peta	P	1,000,000,000,000,000	10^{15}
tera	T	1,000,000,000,000	10^{12}
giga	**G**	**1,000,000,000**	**10^{9}**
mega	**M**	**1,000,000**	**10^{6}**
kilo	**K**	**1,000**	**10^{3}**
hecto	**H**	**100**	**10^{2}**
deca	**da**	**10**	**10^{1}**
NA		**1**	**10^{0}**
deci	**d**	**0.1**	**10^{-1}**
centi	**c**	**0.01**	**10^{-2}**
milli	**m**	**0.001**	**10^{-3}**
micro	**µ**	**0.000001**	**10^{-6}**
nano	**n**	**0.000000001**	**10^{-9}**
pico	p	0.000000000001	10^{-12}
femto	f	0.000000000000001	10^{-15}
atto	a	0.000000000000000001	10^{-18}
zepto	z	0.000000000000000000001	10^{-21}
yocto	y	0.000000000000000000000001	10^{-24}

Primer 2: Keeping a Laboratory Notebook

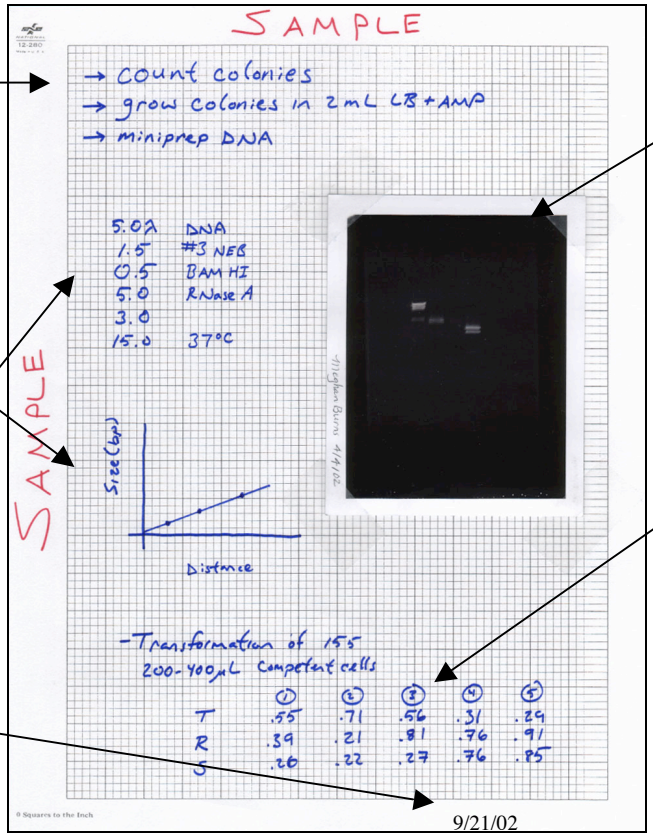
When scientists leave the lab (either to go to lunch or to go to work in another lab), their notebooks stay behind as a testament to what they did there. Other researchers in the lab may wish to know how someone had done a previous experiment, or what the results were from a different trial. Thus the notebook should be organized in such a way as to be intelligible to someone proficient in the field without any input from the author.

You are required to keep a notebook for LB 144. There is graph paper provided for you in the back of this notebook which will be checked to assess your notebook score (so use it as your notebook). BEFORE you leave lab each week, **You MUST get your Notebook stamped and initialed by a TA/LA.** This will be checked & graded throughout the semester and will be a means of taking attendance. Below is shown a sample page from a lab notebook. Although there is one data recorder for the group ALL students will be responsible for transferring the data to their notebooks before leaving lab. Each days entry in your notebook should include the date, the purpose of the experiment, the techniques used, and ORGANIZED data. Your time in lab will be used most effectively if you prepare as many of these elements beforehand as possible. For example, outline what your next experiment is and why you are doing it, write in the protocol or a clear reference to it (be sure to leave room for modifications), and prepare a section to enter data in (what will it look like? Do you need a graph, a table, or something else?). When a protocol is used frequently with only slight modifications, many scientists will type a copy of the protocol in a word-processor and leave blanks in which to fill in important variables (ie: make a protocol for PCR, but leave blanks for things like polymerase concentrations, primer types, etc).

Making a "To Do" list is very useful to do before coming into lab.

Jotting down both numerical as well as pictorial data is extremely useful to you and your TAs when examining your data.

ALWAYS date your work



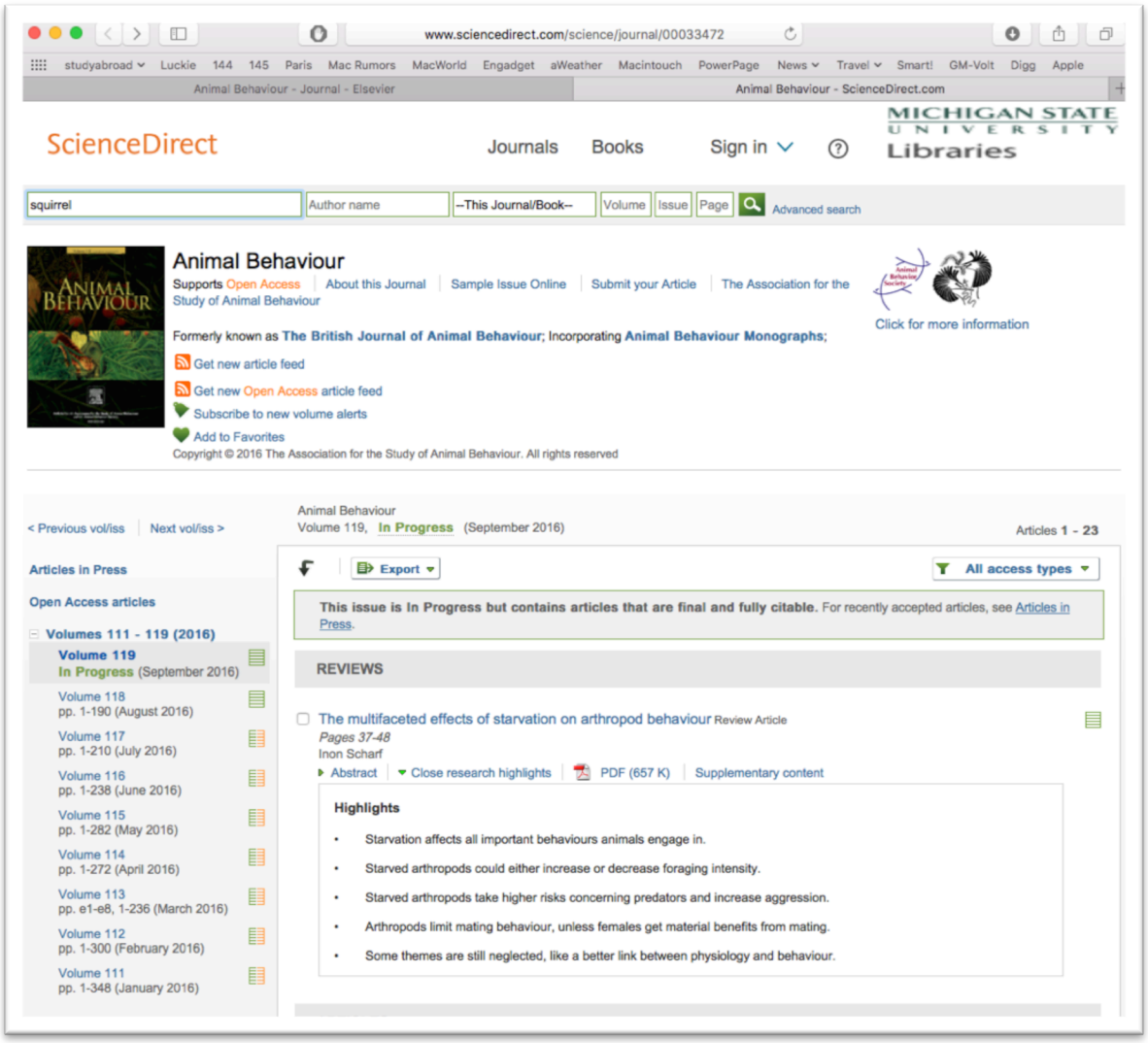
Notice that some material may need to be taped into your lab notebook – this is totally acceptable.

Keeping a record of all data gathered during an experiment at time intervals is absolutely a MUST.

Primer3

How to find published research papers on animal behavior (like squirrels)

Search Google, Google Scholar, and Animal Behavior journal websites



Primer 4: How to read a research paper like a (busy) scientist

By Candace R. Igert

At some point during the semester, you are going to need to read a research paper. If you are striving to do well in this course, you will likely be reading a lot of research papers or journals. Just like everything else in science, there is an effective method to make this less painful and time-consuming. Below is a set of steps an experienced scientist will take when reading a journal or even deciding if it is relevant to what they are looking for. The scientist's goal is getting important information fast. They do not read from beginning to end, but skip around.

Step 1: **A scientist will read the title.** This may sound simple and kind of silly to have it as a step, but the title of a paper can tell you what species/cells were used, what technique was used, what was found, and/or what the research aims were. Sometimes, by this information alone, you can decide if a paper is useful or relevant.

Step 2: **A scientist will then look at the authors and whom they are affiliated with.** This step can help to give credibility to the article or to give you a better idea of what the paper is about. If after reading X amount of journals on CF, you may know that Dr. Smith is an expert on only one aspect of CF or perhaps Dr. Smith works at Harvard, his lends credibility to his/her work. Scientists read slowly and carefully when they determine if the paper is important and/or done by outstanding people.

Step 3: **A scientist will now read critically read the abstract.** At this point, a scientist would read the abstract and glean as much information from it as possible such as the research question, the hypothesis, the predictions, the methods used, the outcome, and how it is interpreted (in other words, what does all of it mean?). All of these aspects are not always apparent or present in every abstract as each journal has different requirements in layout. A great abstract will often share the most important findings and data.

Step 4: **A scientist will read the Introduction if not familiar with the topic.** If the subject that this journal focuses on is not one that you are familiar with, reading the introduction is a way to quickly get yourself up to speed. Perhaps you are familiar with the topic then read the introduction until you catch yourself shaking your head in confirmation or going "Yea, I know this."

Step 5: **A scientist will then go on to look at the figures and tables.** Using the figure and table legends, which are usually pretty extensive and descriptive, a scientist will try to discern what is shown in each figure including what it means in terms of the research aims and interpret data presented in tables. If you come upon something you do not understand by just reading the legend, proceed to Step 5.

Step 6: **A scientist will then read the text to clarify.** When a scientist finds that s/he does not understand a figure or table and needs further explanation, s/he will locate where that figure is referenced in the text and read that portion for

clarification. S/he will first just scan the Results section to find where that figure or table is cited e.g. "(Figure 3)".

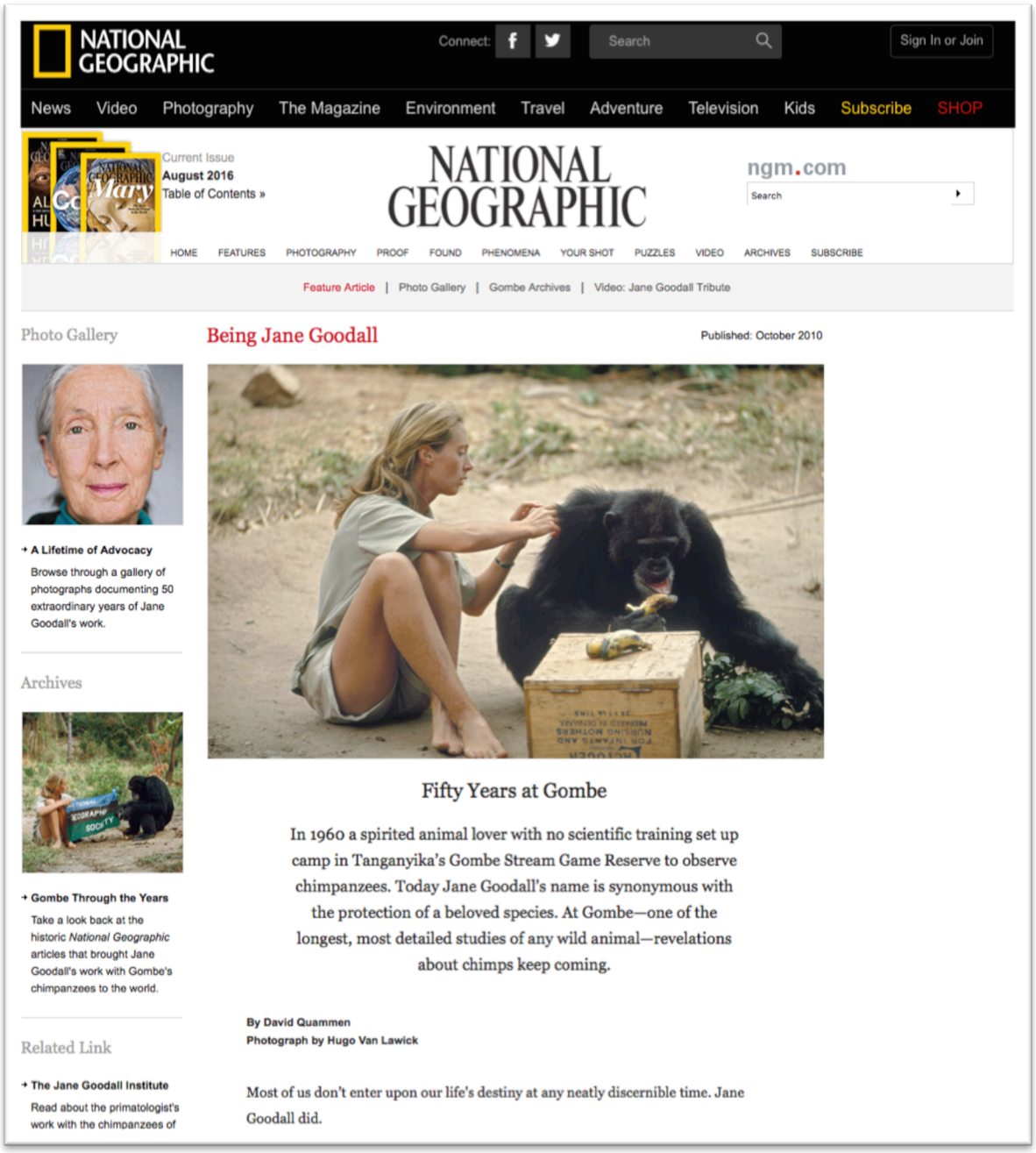
Step 7: **When relevant, a scientist will finally read the discussion.** After reading the title, looking at the authors and their affiliates, critically reading the abstract, and understanding the figures, you may wonder what they concluded from all of that, what they think went wrong/can be fixed in the future, or where they plan to take their research in the future. If this is the case then read the discussion/conclusion.

What information you gain or want from a paper will vary depending on your research needs at that point in time. If you are looking for a primer sequence, then you may skip Step 4 and read the Methods sections to see if their sequence is listed. As you learn how to read papers, you will also learn how to effectively customize the process depending on the occasion.

** Disclaimer: If asked to read a research paper for a class, it is probably best not to only employ this method.

Primer5 BE JANE

Try to learn from Jane Goodall's work *(read more about her online)*



Primer 6

Making video: Nature and wildlife | Life and style | The Guardian 8/7/16

Nature and wildlife

Be it Terry Nutkins, David Bellamy or the late Steve Irwin, everyone has a favourite nature documentary presenter, and most love watching critters from the animal kingdom going about their daily business. (For proof, check out how many people have viewed the legendary "Battle at Kruger" on YouTube.)

Nowadays, there's no reason you can't have a go at creating your own nature documentary. Even with amateur equipment, it's possible to capture extraordinary footage and transform it into an insightful short film without having to book a safari or risk being spat at by a King Cobra. What matters most is to have background knowledge of what you are filming, to know what type of camera moves will suit your subject, and to make the narrative exciting and energetic. Master these basics and you could be well on your way to becoming the next, er, Michaela Strachan.

What to shoot

"Wildlife and nature filming is unpredictable, but the challenge is part of the fun," explains presenter Eleni Andreadis from green.tv, a broadband TV channel for environmental films.

At green.tv, two nature videos are uploaded each week. The footage is no longer than five minutes, it's engaging and packed with information. To create something similar at home, first do some planning and thoroughly research the animal's behaviour. Remember that the subject itself (cute though it may be) won't be enough to create an engaging short film. To make something watchable, you'll also need an informed presenter (which could be yourself) and/or an expert you can interview on screen.

How to prepare

Nature videos are not heavily "storyboarded", but they do need a structure to keep viewers interested. Because of the unpredictablity of the subject , you're going to have to improvise as you shoot, but it's still wise to start with a plan and then adapt it when necessary.

First, think about the aim of your nature video: is it a documentary or a short film? Do you need to interview people? Also, think about the conditions you are likely to be filming in. You'll probably be outside in natural light, so the limitations of your equipment will need to be taken into account.

Planned walks in parks and wetland centres are a good starting point, as the animals are more contained and easier to film. If you are going on a tour, check it's OK for you to film, and factor in the tour's pace. If it's led by an expert, ask if you can interview them. Above all, research everything (and double-check it) before you start filming -

particularly if you are making a more political film about the effects of climate change, say. Without an informed commentary to support your images, the film will be lightweight.

On the shoot

"Let the animal walk out of the shot," advises green.tv's Verity Cowper. "Film the spot you think they are headed towards, and get them coming into and then leaving the frame. This gives your footage a start and end when you come to edit a sequence together - just make sure there's a series of shots at different focal lengths [not necessarily all taken in sequence] edited between these two to avoid the footage being dull for viewers."

The pull focus shot works well in nature documentaries as it helps to put your subject in context and draw in your audience.

If you are using a presenter in the footage, make sure there's a balance between the amount of nature and presenter shown. And if the footage is going online, it really needs to be short and succinct.

Equipment

If you are filming in unreliable weather, you'll probably want to invest in a rain cover for your camera. These are widely available from £70 (creativevideo.co.uk). An external mic for your presenter or interviewee will add finesse.

What not to do

You'll be shooting unpredictable subjects outdoors, so get familiar with your kit. Don't invest in artificial lighting or try techniques you're not confident with. By the time you've worked out the shot, your animal will have vanished out of sight! Also, allow your camera to acclimatise if you're coming in from the cold: you won't see a thing through a steamed- up lens.

Tips and techniques

A quirky tip is to watch Lion King! "Even though it's animated, that film offers a great example of the different focal lengths useful in nature videos," says Verity.

Resources

· green.tv/wwt View the wide range of wildlife and other eco-shorts - and even apply to be a presenter!

· wildlife-film.comInternational site offering training courses, stock footage and more

Primer 7

Getting started with HTML

http://www.w3.org/MarkUp/Guide/Overview.html

by Dave Raggett

This is a short introduction to writing HTML. What is HTML? It is a special kind of text document that is used by Web browsers to present text and graphics. The text includes markup tags such as to indicate the start of a paragraph, and to indicate the end of a paragraph. HTML documents are often referred to as "Web pages". The browser retrieves Web pages from Web servers that thanks to the Internet, can be pretty much anywhere in World.

Many people still write HTML by hand using tools such as NotePad on Windows, or TextEdit on the Mac. This guide will get you up and running. Even if you don't intend to edit HTML directly and instead plan to use an HTML editor such as Netscape Composer, or W3C's Amaya, this guide will enable you to understand enough to make better use of such tools and how to make your HTML documents accessible on a wide range of browsers.

p.s. a good way to learn is to look at how other people have coded their html pages. To do this, click on the "View" menu and then on "Source". On some browsers, you instead need to click on the "File" menu and then on "View Source". Try it with this page to see how I have applied the ideas I explain below. You will find yourself developing a critical eye as many pages look rather a mess under the hood!

For Mac users, before you can save a file with the ".html" extension, you will need to ensure that your document is formatted as plain text. For TextEdit, you can set this with the "Format" menu's "Make Plain Text" option.

This page will teach you how to:
- start with a title
- add headings and paragraphs
- add emphasis to your text
- add images
- add links to other pages
- use various kinds of lists

Start with a title

Every HTML document needs a title. Here is what you need to type:

```
<title>My first HTML document</title>
```

Change the text from "My first HTML document" to suit your own needs. The title text is preceded by the start tag <title> and ends with the matching end tag </title>. The title should be placed at the beginning of your document.

To try this out, type the above into a text editor and save the file as "test.html", then view the file in a web browser. If the file extension is ".html" or ".htm" then the browser will recognize it as HTML. Most browsers show the title in the window caption bar. With just a title, the browser will

show a blank page. Don't worry. The next section will show how to add displayable content.

Add headings and paragraphs

If you have used Microsoft Word, you will be familiar with the built in styles for headings of differing importance. In HTML there are six levels of headings. H1 is the most important, H2 is slightly less important, and so on down to H6, the least important.

Here is how to add an important heading:

```
<h1>An important heading</h1>
```

and here is a slightly less important heading:

```
<h2>A slightly less important heading</h2>
```

Each paragraph you write should start with a tag. The is optional, unlike the end tags for elements like headings. For example:

```
<p>This is the first paragraph.</p>
<p>This is the second paragraph.</p>
```

Adding a bit of emphasis

You can emphasize one or more words with the tag, for instance:

```
This is a really <em>interesting</em> topic!
```

Adding interest to your pages with images

Images can be used to make your Web pages distinctive and greatly help to get your message across. The simple way to add an image is using the <img> tag. Let's assume you have an image file called "peter.jpg" in the same

folder/directory as your HTML file. It is 200 pixels wide by 150 pixels high.

```
<img src="peter.jpg" width="200" height="150">
```

The src attribute names the image file. The width and height aren't strictly necessary but help to speed the display of your Web page. Something is still missing! People who can't see the image need a description they can read in its absence. You can add a short description as follows:

```
<img src="peter.jpg" width="200" height="150"
alt="My friend Peter">
```

The alt attribute is used to give the short description, in this case "My friend Peter". For complex images, you may need to also give a longer description. Assuming this has been written in the file "peter.html", you can add one as follows using the longdesc attribute:

```
<img src="peter.jpg" width="200" height="150"
alt="My friend Peter" longdesc="peter.html">
```

You can create images in a number of ways, for instance with a digital camera, by scanning an

image in, or creating one with a painting or drawing program. Most browsers understand GIF and JPEG image formats, newer browsers also understand the PNG image format. To avoid long delays while the image is downloaded over the network, you should avoid using large image files.

Generally speaking, JPEG is best for photographs and other smoothly varying images, while GIF and PNG are good for graphics art involving flat areas of color, lines and text. All three formats support options for progressive rendering where a crude version of the image is sent first and progressively refined.

Adding links to other pages

What makes the Web so effective is the ability to define links from one page to another, and to follow links at the click of a button. A single click can take you right across the world!

Links are defined with the <a> tag. Lets define a link to the page defined in the file "peter.html" in the same folder/directory as the HTML file you are editing:

```
This a link to <a href="peter.html">Peter's page</a>.
```

The text between the <a> and the is used as the caption for the link. It is common for the caption to be in blue underlined text.

If the file you are linking to is in a parent folder/directory, you need to put "../" in front of it, for instance:

```
<a href="../mary.html">Mary's page</a>
```

If the file you are linking to is in a subdirectory, you need to put the name of the

subdirectory followed by a "/" in front of it, for instance:

```
<a href="friends/sue.html">Sue's page</a>
```

The use of relative paths allows you to link to a file by walking up and down the tree of directories as needed, for instance:

```
<a href="../college/friends/john.html">John's page</a>
```

Which first looks in the parent directory for another directory called "college", and then at a subdirectory of that named "friends" for a file called "john.html".

To link to a page on another Web site you need to give the full Web address (commonly called a URL), for instance to link to www.w3.org you need to write:

```
This is a link to <a href="http://www.w3.org/">W3C</a>.
```

You can turn an image into a hypertext link, for example, the following allows you to click on the company logo to get to the home page:

```
<a href="/"><img src="logo.gif" alt="home page"></a>
```

This uses "/" to refer to the root of the directory tree, i.e. the home page.

Three kinds of lists

HTML supports three kinds of lists. The first kind is a bulletted list, often called an ***unordered list***. It uses the <ul> and <li> tags, for instance:

```
<ul>
  <li>the first list item</li>
  <li>the second list item</li>
  <li>the third list item</li>
</ul>
```

Note that you always need to end the list with the end tag, but that the is optional and can be left off. The second kind of list is a numbered list, often called an ***ordered list***. It uses the <ol> and <li> tags. For instance:

```
<ol>
  <li>the first list item</li>
  <li>the second list item</li>
  <li>the third list item</li>
</ol>
```

Like bulletted lists, you always need to end the list with the end tag, but the end tag is optional and can be left off.

The third and final kind of list is the definition list. This allows you to list terms and their definitions. This kind of list starts with a <dl> tag and ends with </dl> Each term starts with a <dt> tag and each definition starts with a <dd>. For instance:

```
<dl>
  <dt>the first term</dt>
  <dd>its definition</dd>
  <dt>the second term</dt>
  <dd>its definition</dd>
  <dt>the third term</dt>
  <dd>its definition</dd>
</dl>
```

The end tags </dt> and </dd> are optional and can be left off. Note that lists can be nested, one within another. For instance:

```
<ol>
  <li>the first list item</li>
  <li>
    the second list item
    <ul>
      <li>first nested item</li>
      <li>second nested item</li>
    </ul>
</li>
```

```
  <li>the third list item</li>
</ol>
```

You can also make use of paragraphs and headings etc. for longer list items.

HTML has a head and a body

If you use your web browser's view source feature (see the View or File menus) you can see the structure of HTML pages. The document generally starts with a declaration of which version of HTML has been used, and is then followed by an <html> tag followed by <head> and at the very end by </html>. The <html> ... </html> acts like a container for the document. The <head> ... </head> contains the title, and information on style sheets and scripts, while the <body> ... </body> contains the markup with the visible content. Here is a template you can copy and paste into your text editor for creating your own pages:

```
<!DOCTYPE html PUBLIC "-//W3C//DTD HTML 4.01 Transitional//EN"
    "http://www.w3.org/TR/html4/loose.dtd">
<html>
<head>
  <title> replace with your document's title </title>
</head>
<body>

replace with your document's content

</body>
</html>
```

Tidying up your markup

A convenient way to automatically fix markup errors is to use HTML Tidy which also tidies the markup making it easier to read and easier to edit. I recommend you regularly run Tidy over any markup you are editing. Tidy is very effective at cleaning up markup created by authoring tools with sloppy habits. Tidy is available for a wide range of operating systems from the TidyLib Sourceforge site, and has also been integrated into a variety of HTML editing tools.

Getting Further Information

If you are ready to learn more, I have prepared some accompanying material on advanced HTML and adding a touch of style.

W3C's Recommendation for HTML 4.0 is the authoritative specification for HTML. However, it is a technical specification. For a less technical source of information you may want to purchase one of the many books on HTML, for example "Raggett on HTML 4", published 1998 by Addison Wesley. XHTML 1.0 is now a W3C Recommendation.

Best of luck and get writing!

Dave Raggett <dsr@w3.org>

Primer 8: Examples of how to write an email to a researcher

Here are some examples of the style and wording you should go for when emailing a scientist to seek a sample of genomic DNA with your particular mutation.

Your goal is to establish an authentic one-on-one conversation between two peer researchers. Be a scientist not a student. Be sure to send the email from your msu.edu email account. They will look at that first. It establishes credibility.

Dear Dr. X,

I just read your article in The Canadian Journal of Neurological Sciences on dystrophin mutations and seek your advice. I'm currently designing a customized PCR assay to detect the deletion of exon 47 on the dystrophin gene and I'm curious if there is anyone in the field you might recommend I contact to obtain a small sample of genomic DNA with the mutation to serve as a control for testing my assay.

Any help you can send my way would be greatly appreciated.

Sincerely,

Kim Vi
MSU Diagnostics Lab
Michigan State University
vikim@msu.edu

Dear Dr. Fang,

I'm studying the SMN1 gene and just read your 2012 article in Biomedcentral Medical Genetics on SMN1 gene mutations in SMA patients of Chinese descent. I'm currently designing a diagnostic assay for the Arg288Met mutation in SMN1 using PCR. I'm contacting you for advice. Do you know of anyone in the field who I might contact to obtain a small sample of genomic sequence to serve as a positive control in my work?

Any help you could send my way would be greatly appreciated.

Best Regards,

Rajvinder Singh
Research Laboratory of Natural Science
Michigan State University

THE ELEMENTS OF A GOOD HYPOTHESIS

The American Heritage Dictionary defines hypothesis as "*a tentative explanation for an observation, phenomenon, or scientific problem that can be tested by further investigation*". A hypothesis is a possible answer to a question, from which predictions can be made and tested. There can be multiple hypotheses used to answer a single question and for each hypothesis, multiple predictions can usually be made.

The foundation for high quality, biological research is a good hypothesis. A good hypothesis is more than just an educated guess.

THE HYPOTHESIS SCORE CARD…

A good hypothesis must:

1.) explain how or why: provide a mechanism

2.) be compatible with and based upon the existing body of evidence.

3.) link an effect to a variable.

4.) state the expected effect.

5.) be testable.

6.) have at least two outcomes.

7.) have the potential to be refuted.

Hypotheses can be scored based on these elements. When considering a hypothesis, give one point for each of the elements. An *accomplished hypothesis* will have a score of 7. An *incomplete or developing hypothesis* will have a score of 5-6. A score below 5 is an *attempted hypothesis* or *not a hypothesis*. You should use this scoring procedure when developing your own hypotheses or when evaluating hypotheses of others.

Writing Information

Instructions to Authors

Follow these instructions or your paper will be returned to you, and incur late penalties.

One of the learning objectives of your research project in the course is to develop your scientific writing skills. In science, writing is the most important means of communicating research findings. Major scientific findings are rarely kept secret. Instead, scientists share their ideas and results with other scientists, encouraging critical review and alternate interpretations from colleagues and the entire scientific community. In most cases, scientists report the results of their research activities in scientific journals in a standard written format. In this course, you will practice writing using this same standard scientific format and style.

4.0 TIP: Write like a scientist. Write your papers so that anyone who reads your manuscript could not tell it was from a student, but assumes it must have been from a scientist in a lab at MSU.

A scientific paper includes the following: a **TITLE** (statement of the question or problem), an **ABSTRACT** (short summary), an **INTRODUCTION** (background and significance of the problem), a **METHODS** section (report of exactly what you did), a **RESULTS** section (presentation of data), a **DISCUSSION** section (interpretation and discussion of your results), and **REFERENCES** (books and periodicals used). Data is also represented by **FIGURES** and **TABLES**.

Throughout the laboratory, you will practice scientific research and writing. Your papers will be reviewed by the course professor, TAs, and your peers in order to point out your areas of weakness and make suggestions for future improvements. By the time you have completed the course, you will have submitted the equivalent of two full scientific papers. If you are not certain about the level of independence and what constitutes plagiarism in this program, ask your instructor to clarify the class policy. ***Plagiarism will not be taken lightly and will be evaluated by instructors and software at turnitin.com. See syllabus for more info.***

Predictions: Science is not about explanation, but in fact it is about the ability to predict. All scientists must have models or hypotheses that can be used to then make predictions of what will occur. Thus prediction is a very important part of writing you papers. As a result in your early DRAFT1 and DRAFT2 in many cases your predictions may be all the data you have on a certain experiment and thus they should be well supported by papers from the literature. Use future tense when discussing Predictions. In general whether it's in the Abstract, Introduction, Results or Discussion, whenever you discuss something that you predict it should be in the format of:

"We predict..[what].. because..[rationale].. (citation of paper)."

**Note: A hypothesis is different than a prediction. The hypothesis is the model that explains how you believe things are working (e.g. we hypothesize electricity sparks gas in the cylinders of a car engine) while the prediction is what you think should happen during the experiment (e.g. if hydrogen gas is injected into the cylinder of a functioning engine we predict there should be a significant explosion).

Overview of Manuscript Sequence & Format
(This is what professional journals expect)

1. Page one is the Title Page (≤100 characters in title)
2. Page two will have ONLY the Abstract (≤250 words)
3. Page three will start with the Introduction. The Methods, Results, and Discussion sections can follow without starting a new page for each one (although you may start a new page if you are near the bottom of the current page). Clearly label each section with the section headings (ex. Introduction) and who authored it (Written by: Jill Sanders, Revised by: Bob Roberts).
4. The **Introduction** will provide the reader with the background information necessary to understand the rest of the paper.
5. **Methods** section will list materials used (Bought Vitamin C with Rose hips from the General Nutrition Store (GNC) 324 E. Grand River East Lansing MI) how stock solutions were prepared and explain exactly what you did in your research. After reading the Methods section, an incoming student should be able to repeat your work. Reference the original protocol.
6. The **Results** section follows Methods. This section will clearly and succinctly state what you observed upon performing each experiment.
7. The **Discussion** section follows Results. In this section you will discuss the significance of results and how your results relate with research performed by others.
8. The **References** section follows the Discussion. This is a list of the references cited within the paper.
9. Start a new page with the **Figures** section after the References. Figures will be sequentially numbered in the order that they were cited in the Results section (figures are most always cited ONLY in the Results section, not in Methods, not in Discussion). One figure per page with extensive figure legend paragraph ONLY at the bottom of the figure. The first sentence of a figure legend is its title. Follow the title with sentences explaining the figure as if someone did not have the Results section or in fact any other part of the paper available as a reference.
10. The **Tables** section will follow the Figures section. Tables get a title ONLY on the top with some explanation. Tables will be sequentially numbered in the order that they were cited in the Results section (Tables are most always cited ONLY in the Results). One table per page.
11. Figures and tables MUST be created on a computer unless otherwise instructed.
12. After the Tables section, a single white page will follow entitled, **Appendix**. Then append any laboratory notebook pages that indicate signed data for all members (photocopies from your notebook) and a photocopy of the first page of any articles cited and referenced in report.
13. Double space or 1.5 space typeface is required. Preferred font size is 12 point.

***Once returned, rejected papers (like late papers) lose one point in the first 24 hrs grace period but then the penalty becomes more severe: 10% off for 2 days late, 20% off for 3 days, and so on. After 5 days, you will receieve a "0". Unlike late papers, a rejected paper also loses 1 pt per rejection.**

A more detailed description of each section of a scientific paper follows also, review the published papers provided in the course packet for examples. As you write your paper, clearly label each section (except the title page), placing the title of the section on a separate line, centered, bold, but not underlined (like shown below).

Title Page and Title

The title page is the first page of the paper and includes the title of the paper, your name, the course title, your lab time, your lab instructors' names, the due date for the paper, and your groups' website address. The title should be as short as possible and as long as necessary to communicate to the reader the question being answered in the paper. Consider the following titles for a paper that describes the molecular mechanism of an antiviral drug.

1. "Inhibition of Mengovirus Replication by Dipyridamole"
2. "Antiviral Action of Dipyridamole"
3. "A Study Examining the Inhibitory Effects of the Drug Dipyridamole on Mengovirus Replication"

Title 1 is short and communicates the question being investigated. It conveys the mechanism of action (inhibition of replication), the name of the virus being inhibited (Mengovirus), and the name of the drug doing the inhibiting (Dipyridamole). Title 2 is short but too vague for the reader to know the subject matter of the paper. Title 3 is too long. The words "A Study Examining" are superfluous, and "Drug" and is redundant.

Place the title about 7 cm from the top of the title page. Place "by" and your name(s) in the center of the page, and place the course title, lab time, lab instructors' names, due date, and your groups' website address, each on a separate centered line, at the bottom of the page. Leave about 5 cm of white space below this information.

Abstract

The abstract is placed at the beginning of the second page of the paper, after the title page. The abstract summarizes the question being investigated in the paper, the methods used in the experiment, the results, and the conclusions drawn. The reader should be able to determine the major topics in the paper without reading the entire paper. As mentioned previously, predictions are an essential element of science and thus should appear in the Abstract of DRAFT1 and DRAFT2 and in the format: ***"We predict..[what].. because..[rationale].. (citation of paper)."***

Introduction

Start the introduction on page three. The introduction should generally be short, only 4-5 paragraphs in length and focus are background information of the following types:

1. Describe the question and hypothesis being investigated and background on the importance of the topic.
2. Review the background information that will allow the reader to understand the purpose and topics of the paper. There is usually a paragraph on the specimens studied, also one that provides evidence to support the hypothesis posed. A hypothesis is an educated guess; the Introduction should provide the "education." Include only information that directly prepares the reader to understand the question investigated. Most of this information should come

from outside sources, such as scientific journals or books dealing with the topic you are investigating.*

3. In a paragraph state background information on the methods chosen to investigate the hypothesis. Explain how these methods will address the question and describe the predicted outcomes. Why were they chosen?
4. In the last paragraph briefly state a hint of the results and conclusions of the investigations (or predictions). This generally comes only at the very end of the Introduction.

*All sources of information must be referenced and included in the References section of the paper, but the introduction must be in your own words. ***No "quotations" are permitted in any part of the paper.*** Refer to the references when appropriate. As you describe your investigation, include only the question and hypothesis that you actually investigated. It is a good idea to write down each item (question, hypothesis, supporting evidence, prediction) before you begin to write your introduction.

Write the introduction in past tense when referring to elements of your experimental investigation that are completed. When relating the background information, use present tense when referring to another investigator's published work. Use future tense when discussing Predictions.

Methods

The Methods section describes your experiment in such a way that it may be repeated exactly. Make the Methods professional just like in published papers, but target as your audience a student in LB-144. The majority of the information in this section comes from the Procedures or Protocols section of the Laboratory Guide and in your paper, this information should not be a list of steps. Write the Methods section in a paragraph format in past tense. Be sure to include levels of treatment, numbers of replications, and control of treatments. If you are working with living organisms, include the species and the sex of the research organism. Do not include failed attempts unless other investigators may wish to try the technique used. Do not try to justify your procedures in this section of the report.

If you describe an experiment from the lab guide, unless instructed otherwise, you may simply refer to the procedures listed in the guide (and page numbers). Under those circumstances, your Methods section should point out changes in procedure that are not indicated in the Lab Manual. When writing a full Methods section (with no reference to the Lab Guide), write these procedures concisely, but in paragraph form. The difficulty comes as you decide the level of detail to include in your paragraphs. You must determine which details are essential for the investigator to repeat the experiment. For example, if in your experiment you incubated potato pieces in different concentrations of sucrose solution, it would not be necessary to explain that the pieces were incubated in plastic cups labeled with a wax marking pencil. In this case, the molarity of the sucrose solutions, the size of the potato pieces and how they were obtained, and the amount of incubation solution are important items to include

Results

The Results section consists of two components: (1) one or more paragraphs that describe the results of each experiment/test and include the actual data with observations, specific numbers [and units] and math, (2) reference to figures (graphs, diagrams, pictures), and reference to tables.

In referencing figures, remember to number figures and tables consecutively in the order that they are mentioned in this section. Refer to figures and tables within the paragraph as you describe your results, using the word Figure or Table in parentheses, followed by its number, for example, "(Figure 1)." Avoid citing a figure with a full sentence or statement such as, "please see figure 1 for graphed data points" or even just "please see table 2." DO NOT place each figure or table at the end of each paragraph in which it is cited. Place figures and tables after the References section. If you have performed a statistical analysis of your data, such as chi-squared, include this data in the Results section. Explain what calculations you did and the result and direct the reader to the Methods section for more details. Then show an example of the results and refer the reader to a table with all the data.

The most common error a student makes is only making general comments and not including actual data (ie the results) in the Results. Results should include detailed observations (what did you see: odor, texture, etc.) and specific findings (what did you record: 15 grams, 20 degrees C, 20% increase, 32,000 cells etc.). Report your data as accurately as possible in the order that they happened. It is important to have separate paragraphs and topic sentences that introduce the results of each test but do not spend much time discussing the meaning of your findings, save that for the Discussion.

For "***Predicted Results***" - which will be the only focus of Draft 1 and will be included in subsequent drafts - these are what you predict or expect will happen, and these predictions must be based upon the primary literature you have gathered throughout your research on your topic/thesis. When including predictions follow this format: ***"We predict..[what].. because..[rationale].. (citation of a published paper or source)."***

Discussion

The Discussion section is where you will analyze and interpret the results of your experiments. The Discussion should show a possible relationship between observed facts—those observed by you and those observed by others and reported in published research papers. Write as clearly and succinctly as possible. A good Discussion will include the following:

1. Summarize the Introduction and restate the question and hypothesis being addressed.
2. Briefly summarize the results of the experiments. Do not include details regarding methods.
3. Interpret the results. Explain how the results answer the questions posed. State whether your results support or refute your hypothesis. Do not use the word "prove" in your conclusions. Your results will support, verify, or confirm your hypothesis. They also may negate, refute, or contradict your hypothesis. The word prove is not appropriate in scientific writing.
4. Discuss how your results and interpretations relate with previously published research. This will require you to cite outside references. Some may come from the Introduction, while you will also find new references that specifically relate to your findings. You can speculate and propose theoretical implications of your work.

5. Describe weaknesses in experimental design or technical difficulties that arose during the research. Explain how these problems specifically affected the outcome of the research. Any human errors (spills, etc) discussed should have been then corrected by certain steps.
6. Discuss experiments that would be performed if the research were to be continued. Explain how those experiments would contribute to answering the questions addressed by the research.

Figures

All figures should be computer generated. The format of the figure will depend on the type of data collected. Your figures will include mostly photographs and graphs. The photos and graphs must be done in a professional manner and include computer generated labels when appropriate, and always with only one figure per page. Under each photo or graph, there must be a legend paragraph. The legend paragraph will include the Figure number, a title sentence, and a description of what was done in the experiment and shown in different labeled parts of the figure. A reader must be able to understand the general concept of the experiment performed without reading the Methods section. In fact if a student from LB-144 picks up a single piece of paper with one of your figures on it, they should be able to explain to you what that experiment is about from the legend.

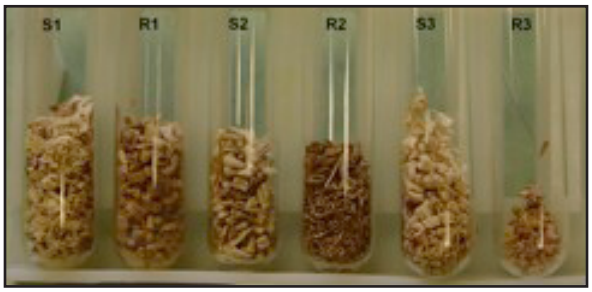


Figure 1. Preparation of corn root and stalk samples for sugar analysis. Samples were cut into units no greater than 4 mm3 using a surgical scalpel. Three stalk samples of 70 grams each and three root samples of 80 grams each were prepared. Root 1 (R1), Root 2 (R2), Root 3 (R3), Stalk 1 (S1), Stalk 2 (S2), and Stalk 3 (S3).

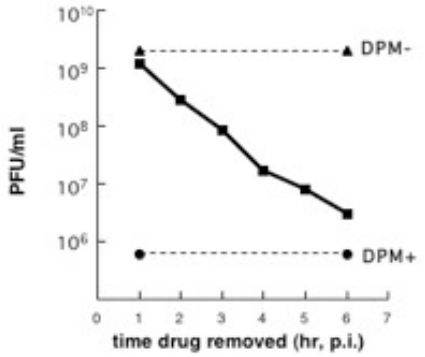


Figure 2. DPM and Virus Yield. HeLa cell monolayers were infected with Mengovirus at a multiplicity of 50 pfu/cell. DPM+ samples had 80 µM DPM (in ethanol) added to the media at the time of infection. DPM- samples were dosed with an equivalent volume of ethanol. Medium from DPM+ cultures was exchanged with drug-free medium at the indicated times. Virus was harvested at 8 hrs PI and the titer determined by plaque assay.

Tables

While Figures are often used in papers (graphs, photographs, gel images) tables are rare. Tables should only be used when all the data being presented cannot be reported in a simple and comprehensible manner in the Results section. The title appears at the top of the table; there is no legend. A footnote may be necessary to clarify an important point in the table.

Table 2. Mengovirus plaque phenotypes in the presence of DPM.

Concentration, μM DPM	Plaque Reduction (%)[a]	Relative Plaque Size[b]
80	100	N/A
60	98	minute
40	93	+
20	68	++
10	25	++
0	0	++++

[a] Values represent the average of two experiments each done in triplicate.

[b] Plaques in the absence of DPM averaged about 2mm in diameter.

Table 1. DNA glycosylases in human cell nuclei.

Enzyme	Size (amino acid residues)	Gene location at chromosome	Altered base removed from DNA
UNG	313	12q23–q24	U and 5-hydroxyuracil
TDG	410	12q24.1	U or T opposite G, ethenocytosine
hSMUG1	270	12q13.1–q14	U (preferentially from single-strand DNA)
MBD4	580	3q21	U or T opposite G at CpG sequences
hOGG1	345	3p25	8-oxo G opposite C, formamidopyrimidine
MYH	521	1p32.1–p34.3	A opposite 8-oxo G
hNTH1	312	16p13.2–p13.3	Thymine glycol, cytosine glycol, dihydrouracil, formamidopyrimidine
MPG	293	16p (near telomere)	3-MeA, ethenoadenine, hypoxanthine

Reference Citation Formatting

A References section lists only those references cited in the paper. You will cite all the references you used when you wrote your paper. In the text of the paper, cite the references using the author's name and publication year. If there are two authors you must state both of their names if there are greater than two authors, state the first authors name followed by et al.

For example: We predict that the 95% alcohol solution will kill all of the NIH-3T3 cells in our culture flask because Smith performed a similar experiment in his paper with another cell type (Smith et al, 1998). In previous research the p58 protein was associated with increased cell growth in C127 cells (Johnson, 2001; Benenson and Kortemeyer, 2003; Haenisch et al, 2006).

Types of Literature:

The vast collection of scientific literature can be generally divided into three categories based on how 'close' they are to the original experiments and descriptions of scientific phenomena. **1) Primary literature**: The bulk of scientific journal articles are primary, meaning that they report the findings of specific experiments or descriptive studies. **2) Secondary literature**: From time-to-time investigators write review articles or books that summarize what is and is not known about a particular topic. Rather than conducting new experiments, these authors rely heavily on the primary literature, therefore these review articles and books are considered a part of the secondary literature. **3) Tertiary literature**: More general texts that summarize what has been reported in review articles comprise the tertiary literature.

Most new research relies heavily on previous work reported in primary literature. However, review articles can be extremely helpful in understanding how your research project fits into the larger scope of scientific investigation, and can be used as a source to locate primary literature references for the topic of interest.

Note that websites were not included in the above description of scientific literature sources. This is because they are not refereed — that is, just about anyone can publish something on the web without some impartial reader reviewing it beforehand. Web pages are often wonderful sources of information, but they can just as often be replete with bad information. At this point, it is very difficult to determine the reliability of web sources and, in general, they should generally only be used as a starting point about a particular topic. ***Thus websites are only allowed as citations in DRAFT1 manuscripts.***

Examples of Proper Citation Formatting for the listings in your Reference section:

Journal articles:

Single Author:

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If the source was published in an online journal do not cite the URL, treat it the same as printed:

Thomas, J. A., M. G. Telfer, D. B. Roy, C. D. Preston, J. J. D. Greenwood, J. Asher, R. Fox, R. T. Clarke, and J. H. Lawton. 2004. Comparative losses of British butterflies, birds, and plants and the global extinction crisis. Science 303: 1879-1881

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Anonymous. 2002. Wisconsin Fast Plants Web Site. http://www.fastplants.org /Introduction/ Introduction.htm, last accessed 7/10/02

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McMillian, V.E. 2001. Writing Papers in the Biological Sciences, 3rd ed. St. Martin's Press, Inc., New York.

Appendix

Note everything you place in your appendix will be discarded during grading.

An Appendix section includes only materials that are not actually required in the paper and could be thrown away with no effect to the paper. Often special extra information is included here. In LB-144 you are expected to place any laboratory notebook pages that indicated signed data for all members (photocopies from your notebook) and a photocopy of the first page of any articles cited and referenced in the report.

Samples: Student Papers

Genotypic Identification of CF Patients with the R553X Mutation using IB3 and S9 Cell Lines and Allele Specific PCR

By: Sarah Bonczyk, Nathan Johns, Elizabeth LeMieux, and Mitch Wood

LB 145 Cell and Molecular Biology
Tuesday 7 PM
Ashley Coulter and Jason Mashni
4/24/2009

http://teamhouselb145.tripod.com/

(Title page written by: Nathan Johns
Revised by: Mitch Wood,
Finalized by: Liz LeMieux)

Abstract

Written by: Nathan Johns
Revised by: Mitch Wood
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The R553X mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) accounts for 0.7% of all cystic fibrosis cases (Hull *et al,* 1993). The mutation involves a single base pair substitution in the 553rd amino acid from CGA (Arginine) to TGA, a stop codon, leading to a truncated protein (Bal *et al,* 1991). Allele specific polymerase chain reaction (ASPCR) was used to determine whether samples of DNA are wild-type, heterozygous, or homozygous for the R553X mutation in the CFTR gene. DNA was extracted from epithelial bronchial cells of known CF patients (Qiagen Inc, 2007). We hypothesized that by controlling annealing temperatures and salt concentrations in the PCR reaction, a single base pair mismatch can be used to determine the presence of the R553X mutation, based on previous PCR diagnostic testing (Chavanas *et al,* 1996). The resulting amplified DNA was then analyzed using agarose gel electrophoresis to determine the genotype of the DNA. We were able to determine whether samples were heterozygous, homozygous wild-type, or homozygous for the mutation by the presence or absence of bands 1,056 base pairs long and based on which forward primer used (Wu *et al*, 1989). Research surveys were distributed and analyzed in order to examine different public opinion on genetic testing between students of various studies at Michigan State University. Results showed no significant difference in opinions on genetic testing among various residential colleges. These tests are significant in helping doctors diagnose cystic fibrosis patients for specific mutations faster and more accurately than previous testing.

Introduction

Written by: Nathan Johns
Revised by: Mitch Wood
Finalized by: Liz LeMieux

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The role of the CFTR protein is to serve as a chloride ion channel in epithelial cells (Rowe *et al*, 2008). Epithelial cells with a CFTR mutation, most often in the lungs, pancreas, and intestines, secrete large amounts of mucus, which builds up and creates complications in the affected tissues (Welsh and Smith, 1995). Mucus buildup in the respiratory tract often leads to pulmonary infection, the most common cause of death in CF patients (Golshahi *et al,* 2008). Current treatments attempt to remove this mucus in the lungs and avoid affection, often by using percussive therapy and antibiotics (Welsh and Smith, 1995).

Over one thousand mutations in the CFTR gene are identified to cause CF, with the ΔF508 mutation, a deletion of three base pairs at position 508, being the most common, and accounting for approximately 70% of all cases (Teem *et al*, 1993). The R553X mutation is the sixth most common, accounts for 0.7% of cases, being most prevalent in German communities (Hull *et al*, 1993). R553X is a nonsense mutation caused by a C to T substitution at the 553rd amino acid. This changes what would normally be arginine to a stop codon (Hull *et al,* 1993). A nonsense mutation is a mutation that causes the DNA sequence to result in a premature stop codon, or a nonsense codon in the mRNA which results in a truncated, incomplete and nonfunctioning protein. In the case of R553X, research has shown that the premature stop codon often results in exon skipping in RNA translation (Aznarez *et al*, 2007). The loss of the exon causes an unstable mRNA of the truncated protein and therefore does not undergo the process of translation (Aznarez *et al*, 2007). The R553X mutation is a class I mutation because of the unstable mRNA synthesized in the nucleus caused by nonsense alleles. (Gambardella *et al.* 2006)

Polymerase chain reaction (PCR) is a technique used to amplify a desired section of DNA (Saiki *et al*, 1988). In PCR, DNA is heated during a denaturing step in order to break the hydrogen bonds between nucleotide bases to separate complimentary 5' and 3' strands. Once

Methods

Written by: Liz LeMieux
Revised by: Sarah Bonczyk
Finalized by: Mitch Wood

Primers

Before the PCR tests were run, primers for the PCR tests were designed using the Cystic Fibrosis Mutation Database. Forward and reverse allele-specific primers that worked with both the wild type and with the mutation were needed. FPrimer1 is a forward primer ending in Guanine, complementary to the wild type amino acid where the mutation should be present. The second of the forward primers, Fprimer2, seeks the mutant type base sequence at the mutation site, base pair number 1789, which results in the primer ending in Adenine. The reverse primer, Rprimer, was designed to bind to the DNA strand 1,022 base pairs past the mutation site between base pairs 2828 and 2811 and was used in both tests. Fprimer1 is 16 base pairs long with the sequence of: GACTCACCTCCAGTT**G** and should properly bind to the wild type gene sequence of **C**AACTGGAGGTGAGTC. Fprimer2, the mutant seeking primer, is also 16 base pairs long with the sequence of: GACTCACCTCCAGTT**A**; the only difference from the previous forward primer is the last base pair, which should properly bind to the mutant R553X gene sequence of **T**AACTGGAGGTGAGTC. The reverse primer that will be used for both tests is 18 base pairs long with the sequence of: CATGAGAGAAGAGAC, which should bind to the gene sequence of TCTGTCTCTTCTCTCATG, which is the same in both mutant and wild type genes. All of the primers are written in 5' to 3'. These primers were ordered from the biological laboratory company Integrated DNA Technologies (IDT).

After the primers were designed, the annealing temperatures at which the primers would bind were determined by first calculating the theoretical melting temperatures of each primer using the following formula:

$$Tm=64.9^\circ C + 41^\circ C \times (\text{number of G's and C's in the primer} - 16.4)/N$$

where N is the length of the primer (Wright *et al,* 2009*)*. The calculated theoretical melting temperatures were: Fprimer1- 45.94°C, Fprimer2 – 43.36°C, and Rprimer – 45.77°C. Based on these calculations, an annealing temperature of 42°C was used in the PCR tests.

DNA Purification

DNA Purification was used to collect DNA from cultured cells of both mutant type and wild type samples to be used in PCR. Two different sources of Human DNA came from Human bronchial epithelial cells from a CF patient without the R553X mutation (IB3 stock cells) while another set of stock cells was found to obtain the R553X mutation, therefore were used as the mutant type test (S9 cells). Each of these stock cells were used to contrast the effectiveness of the allele specific primers. (Gambardella *et al*, 2006) Before beginning purification, all reaction vessels were kept on ice until used and frozen cultured cells were allowed to thaw in a water bath set to 37°C. Then, 200ul of the sample (either human bronchial epithelial cells without the mutation or cells from a CF patient with the mutation, depending on the PCR test being done) was added to a capture column and was incubated at room temperature for 30 minutes. Afterwards, 400ul of DNA Purification Solution 1 was added to the sample. The resulting mixture was incubated for 1 minute at room temperature. After incubation, the mixture was centrifuged for ten seconds at 2,000-12,000 times the force of gravity (xg). The capture column was then transferred into a new waste collection tube. Another 400ul of DNA Purification Solution 1 was added to the mixture and the solution was incubated at room temperature for 1 minute. The solution was then centrifuged again for ten seconds at 2,000-12,000 x g. Then, 200ul of DNA Elution Solution 2 was added and the mixture was centrifuged for ten seconds at 2,000-12,000 x g. The capture column was transferred to a clear DNA collection cube and 100ul of DNA Elution Solution 2 was added. The solution was incubated for ten minutes at 99°C and then centrifuged for 20 seconds at 2,000-12,000 x g. (Quigen Inc. 2007)

PCR

To analyze DNA obtained from the purification process, two different Polymerase Chain Reaction (PCR) tests were designed. Test #1used Fprimer1 and the Rprimer. Test #2 used Fprimer2 and the Rprimer. For each of the tests, the combination of 2.0ul of the target DNA template obtained through DNA purification, 5.0ul 10X PCR buffer, 0.2ul Taq polymerase, 2.0ul forward primer (100 uM), 2.0ul reverse primer (100 uM), 1.0ul 10mM deoxynucleotide building blocks (dNTP) of DNA and 40.4ul water was added to a test tube on ice. The combination was mixed and spun down in a centrifuge. Once the sample was placed in the Labnet thermocycler, the cocktail went through five steps: initial denaturation, denaturation, primer annealing, extension and final extension. In the initial denaturation stage the temperature was raised to 94°

Predicted Results

Authored by: Kevin Werner

In this experiment for the template used in PCR, genomic DNA will be extracted and purified from human cells. Cultured cells from Crohn's Disease patients with the R702W-CARD15 mutation, without the mutation, and heterozygous for the mutation will be obtained from CRISI Inc (Sacramento, CA). For our diagnostic assay, PCR will be used to amplify a DNA segment at a locus containing the R702W mutation on the CARD15 gene that causes a form of Crohn's Disease using a specialized approach for detecting SNPs developed by Hidenobu Yaku (Yaku et al, 2008). The resulting PCR products will be analyzed using agarose gel electrophoresis in either a traditional TBE or fast LB buffer system.

Primer Design

We predict PCR of the genomic DNA will amplify the anticipated products from the R702W locus on the CARD15 gene on chromosome 16 (Figure 1). This will occur because of carefully selected primer design, reactant concentrations, and PCR temperatures and times (Saiki et al, 1998). Thus primers R (annealing at 20,866 base pairs to 20,887 base pairs on the CARD15 gene) and CDF (annealing at 19,866 base pairs to 19,881 pairs on the CARD 15 gene) will amplify a 1021 base pair product, and primers R and WTF (annealing at 19,866 base pairs to 19,881 base pairs on the CARD15 gene) will amplify a 1021 base pair product (Wright, et al. 2010) (Figure 2). Also, the forward primers CDF (forward primer designed to anneal to mutant type template) or WTF (forward primer designed to anneal to wild type template) will not anneal when their

bases are not completly complimentary to the DNA template bases at the respective annealing loci of the primers, preventing amplification from occurring (Schochetman et al, 1988). The forward primers are known as discriminating primers because they are designed to be completely complimentary to either the mutant type or wild type genotype, but not both (Wittwer et al, 1993). Hence it is also predicted that heterozygous DNA will allow for some amplification with both of the discriminating primers. It then follows that amplification of DNA template from the CDF discriminating primer supports the presence of the R702W mutation, which could cause a maladaptive pro-inflammatory response in Paneth cells along with other genetic and environmental factors (Figure 3) (Lala et al, 2003).

We did a preliminary PCR amplification of the 1542 base pair long 16S rDNA locus of *Escherichia coli* to use as a control throughout our experiment (Haffar et al, 2010). The bands we produced during gel electrophoresis exhibited non-specific binding that we were able to reduce by raising the annealing temperature (Figure 4) (Livak et al, 2010). Also, the validity our concentrations of forward primer, reverse primer, dNTPs, buffer, and taq polymerase will supported by these experiments, thus supporting the validity of our Crohn's experiment.

Genomic Purification

In our preliminary research and assay design we found that DNA is extracted from samples in four steps: cell lysis, membrane disruption with a detergent, protein removal, and precipitation (Qiagen 2010) (Figure 5). We predict DNA yield of purified samples will be in the range of 3-8 μg, because this is the normal theoretical yield of

understanding of issues like genetic testing significantly more than any other surveyed group (Figure 6). Using the 1-10 scale for all questions pertaining to genetic testing, all surveyed groups on average responded above the median answer of 5, from which it can be interpreted that Michigan State University students tend to be supportive and knowledgeable about genetics (Table 2).

Discussion

Written by: Mitchell Wood
Revised by: Nathan Johns
Finalized by: Sarah Bonczyk

Experiment Summary

Cystic fibrosis, the most common autosomal recessive disease in Caucasians (Aznarez *et al*, 2007), has dramatic effects on multiple organs, including the lungs, pancreas, intestines, and liver (Welsh and Smith, 1995), due to mutations of the CFTR gene on chromosome seven, causing defects in sodium and chloride transport in epithelial cells (Aznarez *et al*, 2007). The R553X mutation is a specific variation of cystic fibrosis, involving a single base pair substitution at the 1789th base pair in the 553rd amino acid, from cytosine to thymine (Hull *et al*, 1993). The subsequent change from the amino acid arginine to a premature stop codon causes early truncation of the CFTR protein, thus altering the folding sequence (Gambardella *et al.* 2006). Although PCR has been proven effective for diagnosing genetic disorders such as cystic fibrosis (O'Leary *et al*, 1997), the question we are addressing is whether or not a PCR test can be designed to identify this specific mutation. We hypothesized that allele specific primers and a single base pair mismatch could be used to develop an accurate diagnostic test for patients with the R553X mutation using experimentally determined optimal conditions of PCR in terms of annealing temperature, primer concentration, and salt concentration.

In addition to primer design, we bridged the gap between laboratory experiments and the sociology behind diagnosing genetic diseases by surveying student opinion on genetic screening and the effects of genetic diseases on the human race in the long run. In recent years modern medicine has extended the life expectancy of people with cystic fibrosis allowing those affected to live to childbearing age (Ratjen 2008), meaning CF genes are more likely to be passed down to future generations. Samples were taken from Lyman Briggs, James Madison, the College of

Natural Science, and general university students. It was hypothesized that Lyman Briggs students would be more supportive of genetic testing due in part to their background in required integrated studies and their greater understanding of how the frequency of a genetic disease could impact a gene pool (Singer *et al*, 2008).

Original Predictions

By amplifying DNA from IB3 human bronchial epithelial cells from a CF patient and S9 epithelial cells from a leukemia patient via allele specific PCR, the length of the amplified DNA was interpreted through gel electrophoresis to show the presence or absence of the R553X mutation. Two different forward primers, Fprimer1 and Fprimer2, were designed to discriminate between the wild-type and mutant CFTR genes through allele specificity based on a single base pair mismatch on the 3' end. The mismatch was positioned on the 3' end of the primers to more effectively reduce the amplified product by decreasing DNA polymerase and dNTP binding efficiency (Yaku *et al*, 2008). Successful annealing of the primers and the subsequent extension phase was hypothesized to result in a band of 1,056 base pairs, thus indicating a positive test. The lack of a band was hypothesized to indicate a disruption in the extension phase due to the single base pair mismatch (Chavanas *et al*, 1996). A homozygous wild-type genotype was expected to show a band of 1,056 base pairs when using Fprimer1 and show no band when using Fprimer2. In contrast, a homozygous mutant genotype was expected to show a band of 1,056 base pairs when using Fprimer2 and show no band when using Fprimer1. Lastly for heterozygous genotypes, faint bands 1,056 base pairs long were expected to appear in both tests, using either Fprimer1 or Fprimer2 due to the replication of both genotypes during PCR, causing neither set of forward primers to completely discriminate against the specific mutation site (Chavanas *et al*, 1996).

Results and Ultimate Findings

In order to determine optimal PCR conditions, multiple experimental trials were run with adjustments in DNA concentration and primer concentration. In addition, multiple experiments were run to establish the optimal annealing temperature, which can directly affect the annealing rates of designed primers (Elnifro *et al*, 2000) and magnesium chloride ($MgCl_2$) concentration to alter magnesium ion concentrations, which directly affect DNA polymerase activity in PCR (Ignatov *et al*, 2002). The optimal annealing temperature was determined to be 46ºC based off of the calculated primer melting temperatures (see Methods section) and which annealing

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Written by: Mitchell Wood
Revised by: Nathan Johns
Finalized by: Sarah Bonczyk

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Predicted Figures:
Authored by: Kevin Werner

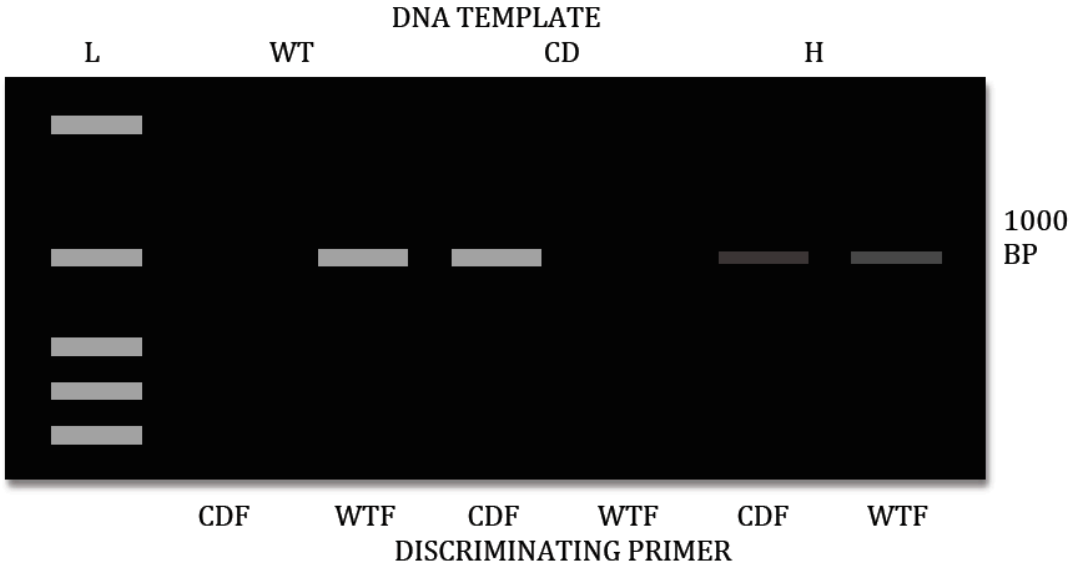

Figure 1 – Predicted results of from amplified products using gel electrophoresis. The non-discriminating oligonucleotide reverse primer, R, is 2% 3'-GTGGCTGCAGGGTTACAACTA-5'. The discriminating oligonucleotide forward primer CDF is 2% 5'-CGGGACGAGGCCGCG-3'. The discriminating oligonucleotide forward primer WTF is 2% 5'-GCGGGACGAGACCGCG-3'. The predicted binding site is on chromosome 16 on the CARD 15 gene from position 19,866 b.p. to 19,881 b.p. for the forward primer CDF, from position 19,866 b.p. to 19,881 b.p. for the forward primer WTF, and from position 20,866 b.p. to 20,887 b.p. for the reverse primer R with respect to the CARD 15 gene. It is predicted there will be a yield of 1.7 billion copies of the target DNA, along with a negligible amount of much larger DNA fragments (a by-product of PCR), and the original DNA template. The denaturing temperature will be set at 95°C, the annealing temperature at 55°C, and the extension temperature at 72°C. An initial denaturing time of two minutes will be used, then the times will be 30 seconds denaturing, 45 seconds annealing, one minute extending, and a final extension of 7 minutes for 30 cycles. The agarose gel contains 10% agarose. All bands are predicted to appear at 1021 base pairs beside the ladder. Bromphenol blue dye will be added to the gel to indicate when the gel electrophoresis is complete. L is the molecular marker or ladder, and the DNA templates WT, CD, and H are 2% DNA purified products from a wild type, mutant, and heterozygous genotype, respectively.

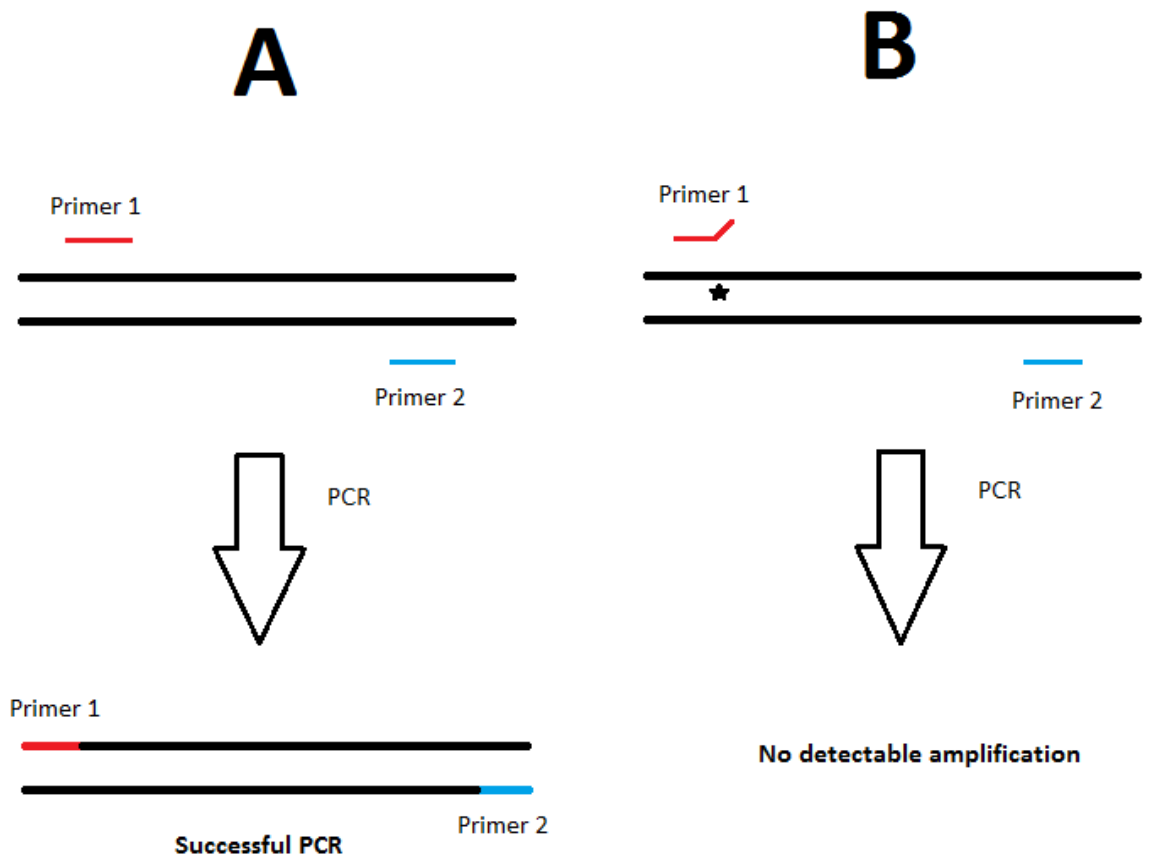

Figure 8. Representation of allele specific PCR. Column A represents wild-type DNA that was extracted using the "generation capture column kit". Column B represents mutant DNA. Primer 1 represents the forward wild-type primer and primer 2 represents the reverse primer. When primer 1 is used with wild-type DNA as seen in column A, PCR completes successfully. This is because the oligonucleotide bases match up with the DNA template and primers. However, when we use primer 1 with mutant DNA as shown in column B, the result is that there is no detectable amplification of DNA.

Figures

Written by: Sarah Bonczyk
Revised by: Liz LeMieux
Finalized by: Nathan Johns

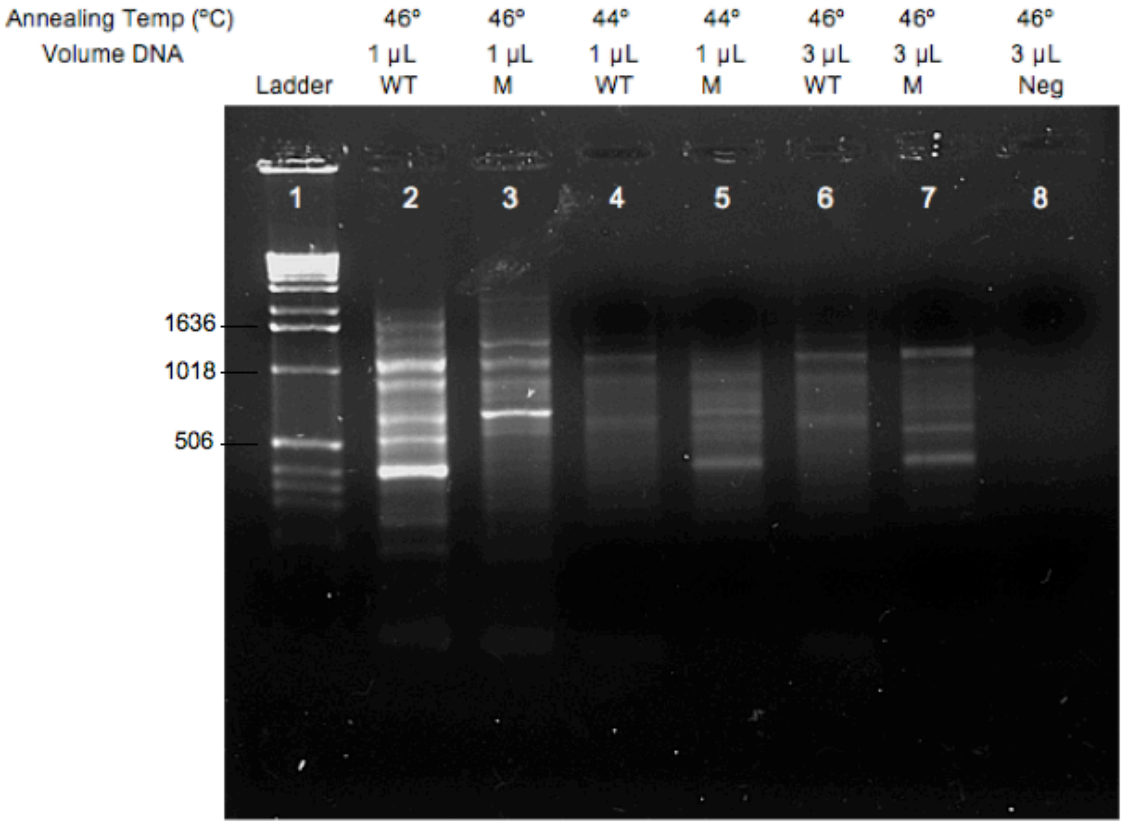

Figure 1: Amplification of DNA segment containing R553X mutation site while varying annealing temperatures and initial S9 DNA volumes. Thermocycling conditions included a 5-minute denaturation at 94 ºC with 30 cycles of 30 s at 94 ºC, 30 s at 46 or 44 ºC, and 60 s at 72 ºC, with a final elongation phase at 72 ºC for 7 minutes. All lanes show non-specific binding. Lanes 2,3,4,6, and 7 show bands near 1018. The targeted region of DNA is 1056 base pairs in length. Lanes using an annealing temperature of 46 ºC and lower initial DNA volumes of 1µL show higher intensity bands than other lanes. From this test it was determined that lower initial concentrations of DNA and an annealing temperature of 46 ºC are optimal for our designed primers.

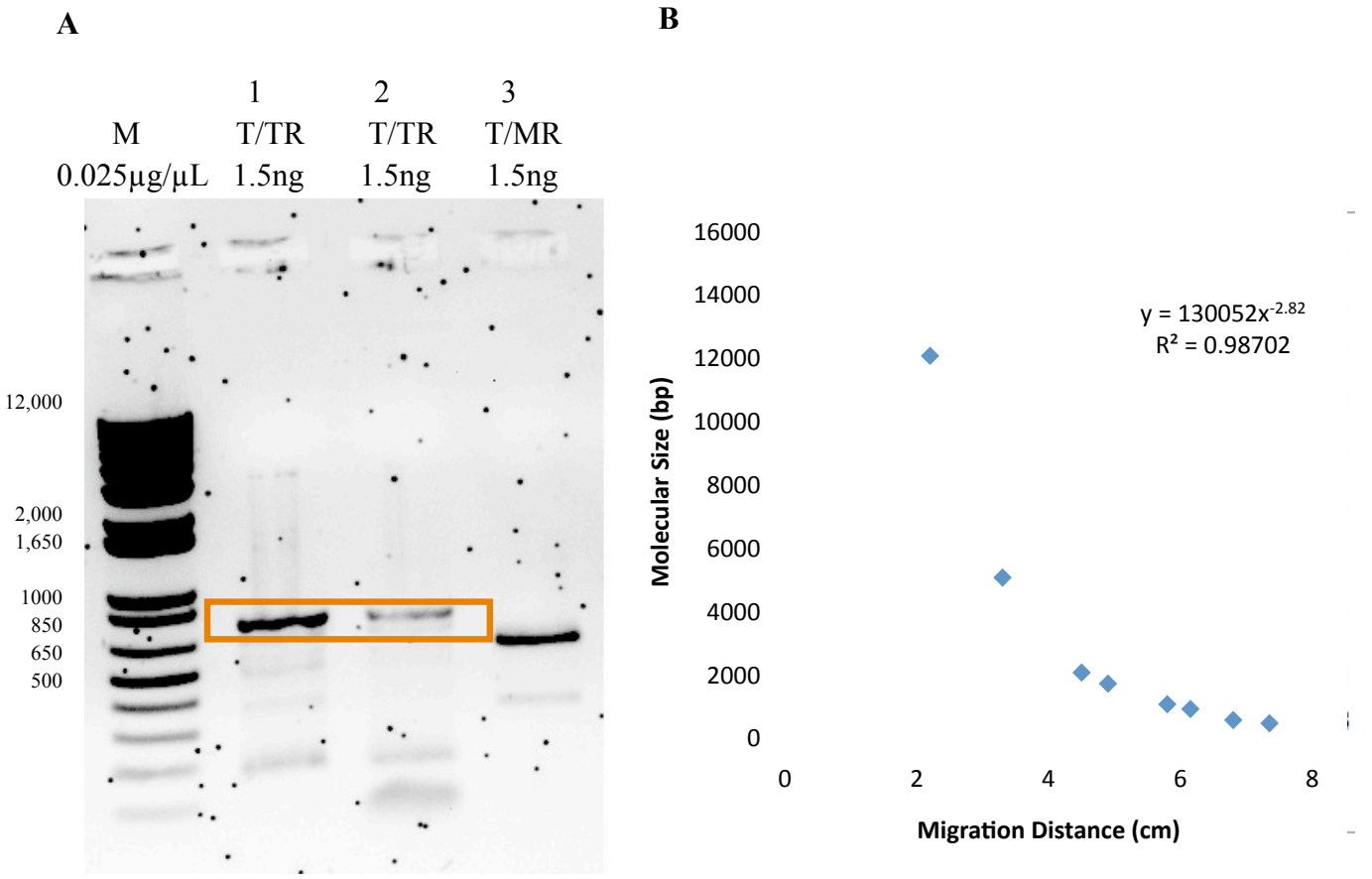

Figure 2. PCR amplification of the DMD gene from human DNA at an annealing temperature of 48ºC. A. After PCR amplification, gel electrophoresis was conducted in a 0.8% TBE gel run at 115V for 30 minutes to detect amplified regions of DNA. M is the molecular marker (1.25ng) 1-Kb Plus Ladder. Lanes 1 and 2 correspond to wild-type DNA samples tested with the wild-type forward/reverse primer set (T/TR). 10µL (1.5ng) of wild-type DNA was added to each of these lanes, and the expected amplification of a 765bp long fragment was observed. Lane 3 corresponds to a wild-type DNA sample amplified with the mutant forward/reverse primer set (T/MR). 10µL (1.5ng) of DNA was added to this lane and a PCR product of 589bp long was observed. For each one of the lanes, non-specific binding was also seen but the expected band was quite distinct.

B. Semi-log plot for 1.25ng of 1-Kb Plus ladder. Each point of the graph depicts the specific distance traveled by each band of the 1-Kb Plus ladder with respect to its well. An R^2 value of 0.98702 ($p<0.05$) was obtained for the logarithmic trend line of the plotted traveled distances. The equation obtained from the trend line was used to calculate the traveled distances of the bands from lanes 1, 2, and 3 to get more accurate band size values. Values of 757.83 ± 8.75bp, 775.33 ± 8.75bp and 589.03 ± 5.21bp were obtained for lanes 1, 2, and 3 respectively.

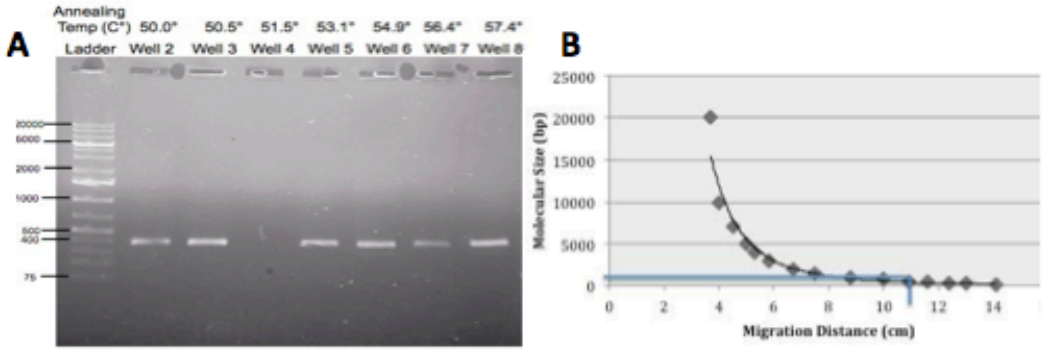

Figure 3: Amplification of lambda RZ gene by PCR and analysis by gel electrophoresis. **(A)** A target DNA sequence of about 400 bp was amplified using PCR in which two primers were used: Rz1R and Rz1F. The PCR cocktails contained 38 µL of nuclease free water, 7 µL of 10X PCR buffer, 1 µL of Taq polymerase, 1µL of dNTPs, 1µL of lambda DNA template, and 1µL of each primer. The cocktails were run in the thermocycler at 95° C for an initial 3 minutes and then cycled between 30 seconds at 95° for the denaturing phase, 30 seconds at the annealing temperature for the annealing phase, and 1 minute at 72° for the elongation phase. The annealing temperatures were set on a gradient from 50° to 58° and 25 cycles were completed. Annealing temperatures are noted above the wells. A 0.8% agarose gel made using TBE (Tris/Borate/EDTA) buffer, agarose, and GloGreen was run at 135V in which 7µL of DNA and 3 µL of loading dye was pipetted into wells 2-8 and 5µL of Fermentas' 1kb Plus ladder was pipetted into well 1. The gel was then observed under an ultraviolet light in order to see the bands created by the DNA product. **(B)** Migration distance vs. molecular size of 1 kb Plus DNA ladder used to analyze PCR products from Rz gene. A target sequence of lambda DNA from the Rz gene was amplified using PCR. An 0.8% agarose gel was made using TBE (Tris/Borate/EDTA) buffer, agarose, and GloGreen. 5 uL of 1 kb Plus ladder was used and analyzed using a semi-log plot shown above in which the x-values represent the distance in centimeters that the bands of the ladder migrated away from the well and the y-values represent the size of the molecules in base pairs. A trend line was added in order to obtain an equation for further analysis. This equation was used to calculate the base pair length of the lambda DNA PCR product in which the distance migrated from the wells was inputted for the x-value of the equation, yielding a y-value representing the base pair length of the product. For the bands produced by the lambda DNA (Figure 5), the base pair length produced was calculated to be 400 bp for all 6 wells yielding a band. The R2 value given in the figure represents the fit of the trend line; 1 represents a perfect fit.

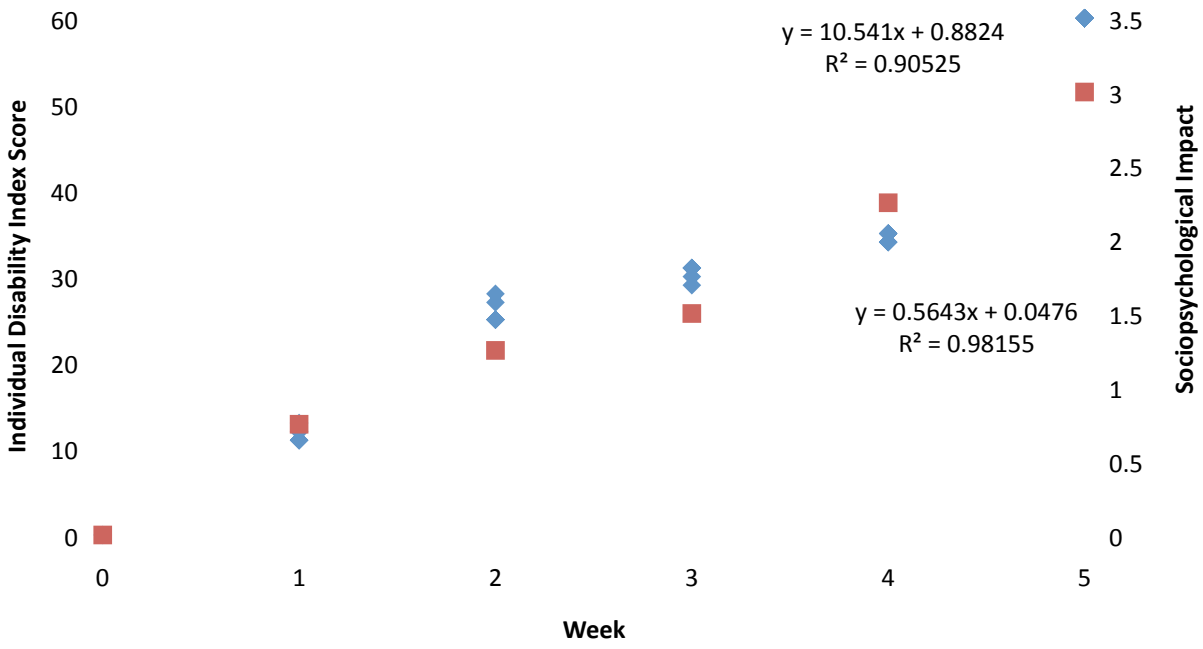

Figure 5. Correlation between the degenerative nature of Duchenne muscular dystrophy and the deteriorating socio-psychological state associated with the disease. Duchenne muscular dystrophy's symptoms were progressively added to each one of the researchers' lifestyles every week for a five-week period. At the end of each week, each researcher completed the Disability Index of Stanford University's Health Assessment Questionnaire (HAQ) in order to gauge the severity of disability that the researchers were living with. The HAQ gives scores between 0 and 60, with zero pertaining to no disability at all and sixty symbolizing complete disability and immobility. In addition to the HAQ, each researcher completed a survey that measured, on a scale of 0 to 3, the socio-psychological impact on the researcher as each symptom was added. A linear regression for the individual Disability Index scores is shown in blue while the linear regression for the Socio-psychological impact test scores is shown in red. An R^2 value of 0.90525 ($p<0.05$) was obtained for the Disability Index score and an R^2 value of 0.98155 ($p<0.05$) was obtained for the socio-psychological impact test.

Table 1. Chi-square test on adverse reactions to CF symptoms in public locations.

Location	Total People Observed	Expected Frequency*	People Observed with Reaction	People Expected with Reaction	$(O-E)^2/E$
Elevator	40	.717	27	28.68	.0984
Cafeteria	25	.717	17	17.93	.0482
Study Lounge	15	.717	9	10.75	.284
Restaurant	10	.717	6	7.17	.191
Total	**90**	**.717**	**59**	**64.53**	X^2=.6216
				n=4	df=3
					p value associated with X^2=.90

*Values representative of the results of question one of Figure 5.

Grading Rubrics

The "Proposal"

What will I have to do?
You will write a title and 2 paragraphs for your group's Proposal (an Introduction & Methods paragraph).

The "Proposal" paper from a group-of-4 will include: A title page with four titles, an Introduction with four paragraphs, and a Methods with four sections. Each student should put their B-PID by their writing so your Title, Intro, Methods can be your grade.

Each student's grade is based 100% on their sections' score. The relevant sections on the DRAFT1 rubric (provided in your Course Pack) will then be used to grade your group's Proposal.

To review, each student will be responsible for authoring:
1. Their own title
2. Their own paragraph (with numerous citations) in the Introduction
3. Their own paragraph/section of the Methods
(we recommend you create a References section, but that is not graded)

TIPS: Your group must coordinate what each person writes about so the final Proposal paper is logical and professional (e.g. each paragraph of the Introduction must discuss different topics). For example: if the first paragraph of the Introduction discusses why beavers are an important part of the economy of Minnesota, the second might then be on a certain communication behavior that is used, the third could be about a Method used to study that behavior and the fourth on a gene that may be important for the animal to perform that behavior. Similar approach in the Methods section, each student should write a paragraph that explains a different Method or part of a method that will be used in your research.

Good news: For the Proposal, your grade is entirely based upon *your* sections! If one of the students in your group never does any of their work, and provides no writing for the final Proposal, it has zero impact on your grade. Just write in pencil on the title page something like "Mr. Hayes had a previous engagement in the Bahamas and sadly was unable to participate in this assignment."

More good news: Blind grading will be used! Be sure to never indicate anyone's name on the manuscript (just B-PID numbers) thus when grading occurs each grader's prior interactions or expectations cannot have an influence on the score. For example, grader likes this section because they liked the author (or the opposite).

Group Inquiry Research Paper DRAFT 1
Due at the start of Lab

****REJECT and HAND BACK IMMEDIATELY if…***
__ The individual authorship of sections is unclear.
__ "Group responsibility" signature page is not completed
__ Submitted manuscript format doesn't not follow "Instructions to Authors"
__ Submitted manuscript has not yet been uploaded to http://turnitin.com/ (or have receipt attached)

ORIGINALITY/CREATIVITY/PROFESSIONALISM: (WORTH 2 POINTS *EC*) ____ ec pts?!

__ **Appendix: (WORTH 2 POINTS)** Do they have copies of signed data *and* the first page of all references?

Title: (WORTH 3 POINTS)
__ Paper gets 2 pts for having a title that says something about **methods** and their **specimen**.
__ Paper gets 1 points if their title predicts anything about their expected **findings**.

Abstract: (WORTH 5 POINTS)
__ Paper gets 3 points for having an abstract that explains well what they **will do and why.**
Including: ___purpose ___hypothesis ___experimental design ___significance in science
__ 2 points if abstract ***predicts*** anything about their expected **findings/results** *with* support from citations.

Introduction: (WORTH 5 POINTS)
__ Paper gets 4 points for having an introduction that tells you some **background** (several paragraphs) about what they plan to do and (one/final paragraph) what they **expect**/predict to see.
__ Paper gets the final 1 point if their introduction talks about **anyone else's research** on this topic or other related reference information from a book or paper etc.
__ Paper gets 1 BONUS point for having an Introduction that makes you **excited** and want to read on.

Methods: (WORTH 5 POINTS)
__ Paper gets 5 points for having a detailed methods section that clearly tells what they will do in a few pages. If you were an LB-144 student and ONLY had the Methods section, **could you repeat** the work?
Including: ___source of materials ___procedures and data analysis ___narrative & in past tense

"Predicted" Results and Figures: (WORTH 5 POINTS)
__ Paper gets 2 points if "Predicted" Results section discusses expected/predicted data that will be collected and supports assertions with rationale and citation of paper. Is each Figure cited properly, does it make sense?
Including: ___outcomes of experiments predicted with support ("We predict ... because... citation/paper")
__ Paper gets 1 point for each "prototype" figure that seems highly appropriate for their investigation.
Including: ___do Figures address research question ___ have a professional appearance ___have long legends with title and extensive description (so a 144 student could explain it with only that page).

Discussion: (WORTH 5 POINTS)
Try to develop a hypothesis that might be tested in your research. Present **why** you have predicted the results you did? The Discussion should be based on **evidence** found in your citations: books, magazines, literature.
__ 1. has an appropriate and interesting TOPIC for the circumstances;
__ 2. has a clear, and preferably original, specific POINT (also known as a THESIS);
__ 3. provides adequate SUPPORT (REASONING and EVIDENCE) for that point; *requires citations*
__ 4. is well ORGANIZED so that the audience can follow the points and examples;
__ 5. employs CLEAR, PRECISE LANGUAGE;
__ 6. is factually ACCURATE and also FAIR, including recognizing objections; *requires citations*
__ 7. is presented in a way that is ENGAGING to the audience;
__ 8. is of a REASONABLE SIZE for the circumstances (not too short or too long);
__ 9. clearly CREDITS OTHERS when their ideas and words are used; *requires citations*
__ 10. Indicates original PREDICTIONS and ultimate findings

The "Half-DRAFT"

What will I have to do?
You will revise 2 paragraphs from your group's Proposal and create a Results paragraph and corresponding figure.

The "Half-Draft" paper your group submits will include: Title page, Introduction, Methods, Results and Figures sections (only a total of 4 Figures, whether predicted or your data or a mix of both, will be allowed).

So to make your Half-DRAFT your group will revise the Title, Intro and Methods you wrote for the Proposal, and add new sections. Each student's grade is based 75% on their individual sections' score, 25% on the final whole paper score. The relevant sections of DRAFT1 rubric will grade the Half-Draft (pts doubled).

Who is assigned to author which part(s)?

Group of 4:
Group works together to pick favorite TITLE (and then make a title page)

PID- responsible for authoring/revising 1st half of INTRO, creating 1 section of RESULTS and limited/permitted to create only 1 Figure (which should be cited in their RESULTS section).

PEDE- responsible for authoring/revising 2nd half of INTRO, 1 section of RESULTS and limited/permitted to create only 1 Figure (cited in their RESULTS section).

DRG- responsible for authoring/revising 1st half of METHODS, 1 section of RESULTS and limited/permitted to create only 1 Figure (cited in their RESULTS section).

LTP- responsible for authoring/revising 2nd half of METHODS, 1 section of RESULTS and limited/permitted to create only 1 Figure (cited in their RESULTS section).

Group of 3:
Group works together to pick favorite TITLE (and then make a title page)

PEDE- responsible for authoring/revising INTRO, 1 section of RESULTS and limited/permitted to create only 1 Figure (which should be cited in their RESULTS section).

DRG- responsible for authoring/revising 1st half of METHODS, 1 section of RESULTS and limited/permitted to create only 1 Figure (cited in their RESULTS section).

LTP- responsible for authoring/revising 2nd half of METHODS, 1 section of RESULTS and limited/permitted to create only 1 Figure (cited in their RESULTS section).

Group Inquiry Research Paper DRAFT 1
Due at the start of Lab

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__ Submitted manuscript has not yet been uploaded to http://turnitin.com/ (or have receipt attached)

ORIGINALITY/CREATIVITY/PROFESSIONALISM: (WORTH 2 POINTS *EC*) ____ ec pts?!

__ **Appendix: (WORTH 2 POINTS)** Do they have copies of signed data *and* the first page of all references?

Title: (WORTH 3 POINTS)
__ Paper gets 2 pts for having a title that says something about **methods** and their **specimen**.
__ Paper gets 1 points if their title predicts anything about their expected **findings**.

Abstract: (WORTH 5 POINTS)
__ Paper gets 3 points for having an abstract that explains well what they **will do and why.**
Including: ___purpose ___hypothesis ___experimental design ___significance in science
__ 2 points if abstract ***predicts*** anything about their expected **findings/results** *with* support from citations.

Introduction: (WORTH 5 POINTS)
__ Paper gets 4 points for having an introduction that tells you some **background** (several paragraphs) about what they plan to do and (one/final paragraph) what they **expect**/predict to see.
__ Paper gets the final 1 point if their introduction talks about **anyone else's research** on this topic or other related reference information from a book or paper etc.
__ Paper gets 1 BONUS point for having an Introduction that makes you **excited** and want to read on.

Methods: (WORTH 5 POINTS)
__ Paper gets 5 points for having a detailed methods section that clearly tells what they will do in a few pages. If you were an LB-144 student and ONLY had the Methods section, **could you repeat** the work?
Including: ___source of materials ___procedures and data analysis ___narrative & in past tense

"Predicted" Results and Figures: (WORTH 5 POINTS)
__ Paper gets 2 points if "Predicted" Results section discusses expected/predicted data that will be collected and supports assertions with rationale and citation of paper. Is each Figure cited properly, does it make sense?
Including: ___outcomes of experiments predicted with support ("We predict ... because... citation/paper")
__ Paper gets 1 point for each "prototype" figure that seems highly appropriate for their investigation.
Including: ___do Figures address research question ___ have a professional appearance ___have long legends with title and extensive description (so a 144 student could explain it with only that page).

Discussion: (WORTH 5 POINTS)
Try to develop a hypothesis that might be tested in your research. Present **why** you have predicted the results you did? The Discussion should be based on **evidence** found in your citations: books, magazines, literature.
__ 1. has an appropriate and interesting TOPIC for the circumstances;
__ 2. has a clear, and preferably original, specific POINT (also known as a THESIS);
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__ 7. is presented in a way that is ENGAGING to the audience;
__ 8. is of a REASONABLE SIZE for the circumstances (not too short or too long);
__ 9. clearly CREDITS OTHERS when their ideas and words are used; *requires citations*
__ 10. Indicates original PREDICTIONS and ultimate findings

The "Draft1" final paper

What will I have to do?
You will take full responsibility for generating a specific section(s) to create your group's final paper.

The "Draft1" paper your group submits will include: Title page, Abstract, Introduction, Methods, Results, Figures, and References sections (in the APPENDIX you just need to provide your graded Half-Draft so we can compare your original version to this one).

Use PIDs not names to indicate who was responsible for each section of the manuscript. But please do *not* keep PIDs associated with each and every paragraph of sections, follow authorship used by the student sample paper in the Course Pack.

Each student's grade is based 50% on their individual sections' score, 50% on the final whole paper score. The relevant sections of DRAFT1 rubric will grade the final paper (pts doubled to 60).

Who is assigned to author which part(s)?

Group of 4:
PID- responsible for authoring/revising all FIGURES.

PEDE- responsible for authoring/revising METHODS.

DRG- responsible for authoring/revising RESULTS

LTP- responsible for authoring/revising TITLE PAGE, INTRODUCTION and REFERENCES.

Group of 3:
PEDE- responsible for authoring/revising METHODS.

DRG- responsible for authoring/revising RESULTS and FIGURES.

LTP- responsible for authoring/revising TITLE PAGE, INTRODUCTION and REFERENCES.

Group of 2:
In a group of 2 one student is the **PID** & **PEDE**, the other is the **DRG** & **LTP**. Hence follow assignments for a group of 4.

Note: Individual responsibilities for the making of your 5-minute-long documentary film are indicated on page where the Roles are described in the Course Pack.

Group Inquiry Research Paper DRAFT 1
Due at the start of Lab

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__ **Appendix: (WORTH 2 POINTS)** Do they have copies of signed data *and* the first page of all references?

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__ Paper gets 2 pts for having a title that says something about **methods** and their **specimen**.
__ Paper gets 1 points if their title predicts anything about their expected **findings**.

Abstract: (WORTH 5 POINTS)
__ Paper gets 3 points for having an abstract that explains well what they **will do and why.**
Including: ___purpose ___hypothesis ___experimental design ___significance in science
__ 2 points if abstract ***predicts*** anything about their expected **findings/results** *with* support from citations.

Introduction: (WORTH 5 POINTS)
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Including: ___source of materials ___procedures and data analysis ___narrative & in past tense

Results and Figures: (WORTH 5 POINTS)
__ Paper gets 2 points if Results section discusses data that was collected and supports assertions with rationale. Is **e**ach Figure cited properly, does it make sense?
Including: ___outcomes of experiments clear __ Paper gets up to 1 point for each novel figure that seems appropriate for their investigation.
Including: ___do Figures address research question ___ have a professional appearance ___have long legends with title and extensive description (so a 144 student could explain it with only that page).

Discussion: (WORTH 5 POINTS)
Try to develop a hypothesis that might be tested in your research. Present **why** you have predicted the results you did? The Discussion should be based on **evidence** found in your citations: books, magazines, literature.
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__ 2. has a clear, and preferably original, specific POINT (also known as a THESIS);
__ 3. provides adequate SUPPORT (REASONING and EVIDENCE) for that point; *requires citations*
__ 4. is well ORGANIZED so that the audience can follow the points and examples;
__ 5. employs CLEAR, PRECISE LANGUAGE;
__ 6. is factually ACCURATE and also FAIR, including recognizing objections; *requires citations*
__ 7. is presented in a way that is ENGAGING to the audience;
__ 8. is of a REASONABLE SIZE for the circumstances (not too short or too long);
__ 9. clearly CREDITS OTHERS when their ideas and words are used; *requires citations*
__ 10. Indicates original PREDICTIONS and ultimate findings

Characteristics of a 4.0 Final Manuscript

1. Reads like a scientist wrote it. The author is clearly well-read on the topics they studied, understands their goals and methodologies and communicates that well in a concise, smart, composition.
2. References section contains scientific research papers, recent books and news articles.
3. Figures looks professional with clear and detailed legends.
4. Introduction and Discussion sections have numerous citations and they are used regularly every few sentences. All sections are clearly broken up into sub sections, with sub titles, that focus on each step of the project (such as: Quantitation of DNA Found, DNA purification, PCR analysis).
5. Each step in the research project is addressed individually and thoroughly with context "why and how it was done" as well as evidence "photographs," data sets, mathematical analysis.
6. Error analysis is scientific and thoughtful; citing difficulties found by other researchers in publications, limitations or possibilities that explain problems in data collection.

Characteristics of a 0.0 Final Manuscript

1. Reads like a high school student wrote it. The author is clearly has not read about the topics they studied (even material presented in the course pack), and does NOT understand their project or communicate well in a composition.
2. References section contains anonymous websites and citations are not complete.
3. Figures looks incomplete with short confusing legends.
4. Introduction and Discussion sections have very few citations and all sections are just long paragraphs with no topic sentences and no sub-headings.
5. Each step in the research project is NOT addressed individually and the author seems only focused on quickly being done.
6. Error analysis is trivial where all the human error items listed could easily have been fixed by using controls or doing replications (or even coming to lab more than once a week and paying attention).

(for) Presentations

Group _____

Rubric for scoring Proposal Talk/Movie (LB144)

_____ [P/F/percent] **LITERATURE** RESEARCH: _____: Student name _____

We used Google Scholar and PubMed to search research papers and reviews on [insert behavior here] and this particular related gene. So far we have found X research papers and Y books that discuss this behavior in ... The first paper is entitled _____ and it demonstrates _____. The second paper is a review ….. We found two research papers where the authors studied genes related to this and found...

_____ [P/F/percent] BACKGROUND ON **BEHAVIOR** _____: Student name _____

We read papers _____, _____, and _____ and found that [insert communication behavior here] has actions like _____, and is communication of ____ type, and the behavior is documented with these strategies. We propose to study it in this animal _____ this way...

_____ [P/F/percent] METHODS & capturing behavior _____: Student name _____

We propose to use smartphone video and boom mic audio to capture this known communication behavior in our _____ animals _____ using this/these method(s) from these published approaches _____, and _____. To further connect our animal research with humans, we propose to study a homologous behavior of _____ and ____ in humans and collect sociological data using these professional published instruments found in this paper _____.

_____ [P/F/percent] METHODS for **genetic** basis : _____: Student name _____

We read the about this gene which may be connected to this behavior. The full genomic sequence of our gene is available here _____. Here's an image of the DNA sequence. We can also use these published DNA primer sequences from this paper (shown in our slide) to amplify it by PCR using these methods. Once we know the base sequence of the primer we can calculate it's expected annealing temperature using these methods.

_____ [P/F/percent] METHODS use of **CONTROLS**: _____: Student name _____

As a "experimental control" we will be sure that _____. We have controls in the behavior studies of animals and humans as well as in the molecular PCR study of our gene. For example, we plan to use positive controls like ….._____ and negative ones for example _____. The role of the controls are these....

Verbal Status Report Grading Rubric (Talk is 10 minutes in length, individually graded)

Assignment Explanation: Each person is responsible for only ONE figure in both the talk. IMPORTANT the figure on your slide should look identical to the same figure in Half-Draft paper, except on the slide you should have almost zero figure legend (ie nearly none of the text). Slides should contain both the most relevant actual data figures to represent what is done [or in progress] and predicted figures to represent precisely what still must be completed.

Student Names: Group Name:

Figure 1: Student _____ Topic _____

Evaluate Slide (visual) _____ Score _____

Evaluate Explanation (verbal) _____ Score _____

Figure 2: Student _____ Topic _____

Evaluate Slide (visual) _____ Score _____

Evaluate Explanation (verbal) _____ Score _____

Figure 3: Student _____ Topic _____

Evaluate Slide (visual) _____ Score _____

Evaluate Explanation (verbal) _____ Score _____

Figure 4: Student _____ Topic _____

Evaluate Slide (visual) _____ Score _____

Evaluate Explanation (verbal) _____ Score _____

Figure 5: Student _____ Topic _____

Evaluate Slide (visual) _____ Score _____

Evaluate Explanation (verbal) _____ Score _____

Grading Scale:

Pretty Good: Average work, respectable, middle of the class.

Excellent. It is impressive work, top of the class, and the work was done extremely well but nothing beyond what is expected.

Most Excellent. Every detail of the work was done extremely well and they found additional papers and evidence beyond what they were told.

Outstanding. It has the 3.0, 3.5-level elements + student impresses instructor with how much/well they did the work. They taught Prof something.

Handouts

JClub papers: Research papers for Journal Clubs

for events occurring farther apart in time. Coallocation is not limited to linking memories at encoding. Memory recall may engage a similar process to link new with old memories. We trained mice on event 1, 2 days before event 2. Event 2 memory was enhanced if event 1 was recalled 6 hours, not 24 hours, before event 2 (Fig. 4D). Here, we find that excitatory-inhibitory balance determines whether memories are bound or, alternately, segregated in the LA. More broadly, these principles provide a foundation for understanding how memories are organized within associative networks.

Note added in proof: During final preparation of this manuscript, a notable study showing time-limited coallocation of hippocampal memory traces was published (*25*).

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/353/6297/383/suppl/DC1
Materials and Methods
Figs. S1 to S9
References (*26*–*43*)

11 December 2015; accepted 17 June 2016
10.1126/science.aaf0594

BEHAVIORAL ECOLOGY

Reciprocal signaling in honeyguide-human mutualism

Claire N. Spottiswoode,[1,2]* Keith S. Begg,[3] Colleen M. Begg[3]

Greater honeyguides (*Indicator indicator*) lead human honey-hunters to wild bees' nests, in a rare example of a mutualistic foraging partnership between humans and free-living wild animals. We show experimentally that a specialized vocal sound made by Mozambican honey-hunters seeking bees' nests elicits elevated cooperative behavior from honeyguides. The production of this sound increased the probability of being guided by a honeyguide from about 33 to 66% and the overall probability of thus finding a bees' nest from 17 to 54%, as compared with other animal or human sounds of similar amplitude. These results provide experimental evidence that a wild animal in a natural setting responds adaptively to a human signal of cooperation.

In 1588, João dos Santos, a Portuguese missionary in Sofala (in present-day Mozambique), often noticed a small bird flying through cracks in the walls of his mission church and nibbling wax from the candlesticks within. This kind of bird, he wrote, had another peculiar habit of leading men to bees' nests by calling and flying from tree to tree. After the men harvested the honey, the birds would eat the wax combs left behind (*1*). We now know this species to have been the greater honeyguide *Indicator indicator* and dos Santos's description to have been accurate. Honeyguides eat beeswax and know where bees' nests are located; humans can subdue the bees and open the nest using fire and tools, thus exposing beeswax for the honeyguides and honey for the humans (*2*). This interaction remains an extremely rare example of mutualism between free-living wild animals and our own species. Here we show that it is also a specific example of reciprocal communication between birds and humans.

Greater honeyguides (Fig. 1A) seeking a human collaborator approach people and give a loud chattering call (audio S1). This call is distinct from their territorial song and is accompanied by referential gestures (*3*): the bird flies from tree to tree in the direction of the bees' nest until its human follower finds the nest (*2*, *4*). The honeyguide thus directs a signal of the bees' nest location toward humans, and the honey-hunters use this signal to their mutual advantage. In this study, we ask whether honeyguides in turn exploit specialized signals directed at them by humans. We studied greater honeyguides (hereafter "honeyguides") in the Niassa National Reserve in northern Mozambique. This region has been noted for its honey and beeswax production at least since Arab trading times centuries ago (*5*, *6*). The local Yao people still harvest wild honey using traditional methods, and this practice remains economically important.

[1]Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK. [2]FitzPatrick Institute of African Ornithology, Department of Science and Technology–National Research Foundation (DST-NRF) Centre of Excellence, University of Cape Town, Rondebosch 7701, South Africa. [3]The Ratel Trust, Niassa Carnivore Project, Private Bag X18, Rondebosch 7701, South Africa.
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First, we confirmed that in northern Mozambique, honeyguides give reliable information to human honey-hunters. To test whether guiding behavior accurately indicates the direction of bees' nests and leads to their successful discovery by humans, we trailed honey-hunters following honeyguides and tracked our movements via GPS. A guiding event was defined as a bout of guiding by an individual bird, sometimes involving consecutive journeys to different bees' nests. Each guiding event probably involved a different individual honeyguide, as the study area was 230 km^2, and the home ranges of individual honeyguides that we measured using radio telemetry did not exceed 1 km^2 and overlapped with one another (*7*) (fig. S1). 75.3% of guiding events led to the successful discovery by humans of at least one bees' nest [mean ± SE = 1.00 ± 0.08 nests; range = 0 to 3 nests; n = 97 events, excluding controls in the experiment discussed below (*7*)]. 94.6% of nests shown belonged to the honeybee *Apis mellifera*, and the rest to stingless bee species (*7*). Nests were located 0 to 832 m (median = 152 m, n = 84 nests, only considering the first nest per guiding event) from the point where guiding began. Figure 1B shows that the birds' initial flight direction accurately signaled the ultimate location of the bees' nest, corroborating a classic study from Kenya (*2*). Overall, 74.5% of bees' nests found by humans (n = 149) involved the help of a honeyguide.

Second, we asked whether the signals used by human honey-hunters provide reliable information to honeyguides. Honey-hunters seeking honeyguides often announce their presence with unspecialized sounds such as shouting and chopping wood (*4*, *8*). In some parts of Africa, however, humans also make specialized vocalizations used only when hunting honey. In the Niassa National Reserve (and, more widely, in northern Mozambique and adjacent southern Tanzania), Yao honey-hunters seeking and following honeyguides produce a loud trill followed by a grunt: "brrrr-hm" [audio S1; see (*9*) for a melodious whistle used in the same context by the Hadza people of northern Tanzania]. To confirm that "brrrr-hm" is a specialized honey-hunting sound, we interviewed 20 Yao honey-hunters, all of whom reported that they used this specific sound when hunting honey but in no other context. When

asked why, they reported that they learned it from their fathers and that it is the best way of attracting a honeyguide and maintaining its attention. Therefore, this sound has the potential to reliably signal to honeyguides that a prospective human partner is specifically seeking honey and has the tools, skills, and time to open a bees' nest, which many humans do not. A payoff to the bird reliably results from interacting with such a partner, because if a bees' nest is harvested then wax is left behind, either as combs containing no honey or as chewed lumps spat out by honey-hunters.

Finally, we examined whether honeyguides associated this vocal signal with a higher chance of a payoff from cooperation. If so, then honeyguides should be more likely to initiate collaboration with humans producing this honey-hunting sound rather than other sounds. To test this, we carried out 72 15-min experimental transects simulating honey-hunting forays, in which an author and two local honey-hunters walked while playing back one of three acoustic cues every 7 s at consistent amplitude using a calibrated speaker: (i) a control human sound (either the Yao words for "honeyguide" and "honey" or the honey-hunter's name, alternated among transects); (ii) a control animal sound (either the song or the excitement call of the ring-necked dove, *Streptopelia capicola*, alternated among transects); or (iii) the specialized "brrrr-hm" honey-hunting sound [see (*7*) for details and audio S2 to S4 for examples]. Each transect used a distinct playback exemplar recorded from a different individual person or bird. Honeyguides have never been confirmed to guide any species besides humans (*10*). We conducted this experiment during the hot dry season; the average shade air temperature at the end of each transect was 31°C (range = 23° to 38°C). Therefore, we also tested the effects of temperature, trial time relative to sunrise or sunset, and morning versus afternoon [details in (*7*)].

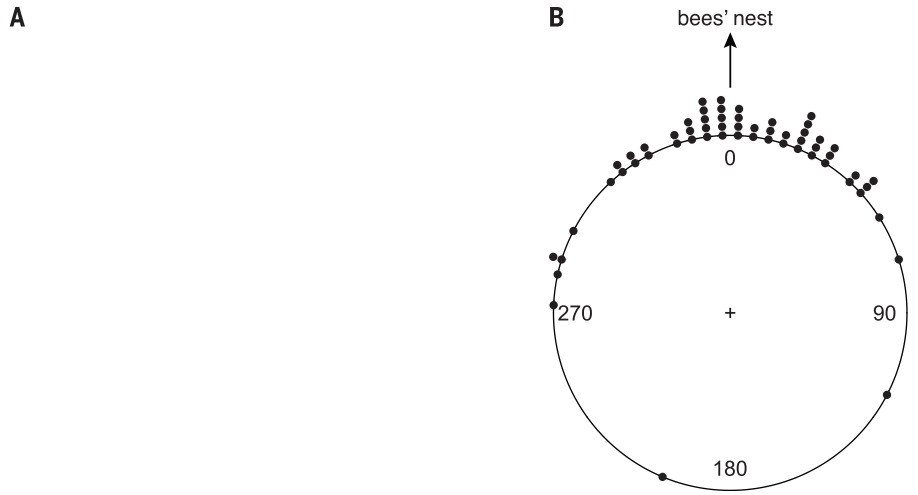

Fig. 1. Greater honeyguides accurately lead humans to bees' nests. (**A**) A Yao honey-hunter and a wild, free-living honeyguide. (This bird was captured using a researcher's mist-net and is neither tame nor habitually captive.) (**B**) Accuracy of honeyguide initial guiding behavior in relation to direction of successfully located bees' nests. Points represent the difference in bearing between initial guiding trajectory over the first 40 m of travel and the ultimate direction of the bees' nest (here set at 0) and are binned into 5° intervals. Each point represents a journey (n = 58 journeys) to a separate bees' nest that was at least 80 m away from the point where guiding began. Sometimes a honeyguide led humans to more than one nest consecutively (n = 50 guiding events). The circular distribution is unimodal (Rayleigh test, $P < 0.001$) with a mean of 1.7° (95% confidence interval includes zero: 352.3° to 11.1°), showing that honeyguide behavior offers reliable directional information to humans.

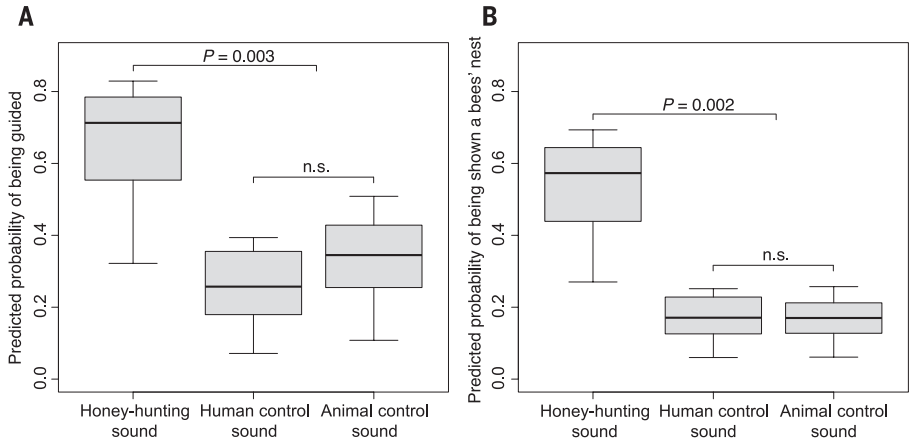

Fig. 2. Probability of a successful mutualistic interaction, in relation to experimentally given acoustic cues. Values are predicted probabilities of (**A**) being guided by a honeyguide and (**B**) being shown a bees' nest on a 15-min search, derived from a logistic model of data from experimental transects and accounting for time of day (minutes from sunrise to sunset). Boxes show medians and quartiles; whiskers show ranges (n = 24 trials per treatment group; P values show planned comparisons; n.s., not significant).

We were guided by a honeyguide on 30 of 72 transects. Transects accompanied by the honey-hunting call had a 66.7% probability of eliciting guiding from a honeyguide, which was significantly greater than that for transects accompanied by the human control sounds (25%) or animal control sounds (33.3%) (Fig. 2A; planned comparison with controls: estimate ± SE = 1.13 ± 0.38, Z = 2.96, P = 0.0031). The probability of guiding did not differ between the two control treatments (estimate ± SE = 0.25 ± 0.33, Z = 0.76, P = 0.45). The best model also included the time relative to sunrise or sunset as a covariate (probability of being guided weakly decreased closer to the middle of the day: estimate ± SE = −4.34 ± 0.20, Z = −2.13, P = 0.034) and, overall, explained 25% of the variance in probability of being led by a honeyguide.

Once a honeyguide initiated guiding behavior, we followed it while continuing to play back the acoustic treatment, while the honey-hunters searched visually for bees' nests [see (*7*) for measures taken to encourage and validate equal search effort]. This revealed that honeyguides tended to cease guiding behavior more often when either of the two control sounds was produced, resulting in no bees' nests being found. Of those transects on which we were led by a honeyguide, we found a bees' nest for 81.3% when accompanied by the honey-hunting sound, compared with 66.7 and 50.0% when accompanied by the human and animal control sounds, respectively. Overall, the honey-hunting sound resulted in a 54.2% predicted probability of finding a bees' nest (Fig. 2B; planned comparison with controls: estimate ± SE = 1.21 ± 0.39, Z = 3.14, P = 0.0017) compared with 16.7% for each of the control sounds (planned comparison between controls: estimate ± SE = 0.03 ± 0.39, Z = 0.08, P = 0.94). Thus, production of the honey-hunting sound more than tripled the probability of finding a bees' nest during a standardized 15-min search accompanied by an acoustic cue. This finding experimentally validates the honey-hunters' claims that the honey-hunting sound improves their foraging success.

Honeyguides might respond more to humans producing the honey-hunting sound either because they recognize and prefer it or because they are simply more likely to hear it versus control sounds. If the latter is true, then honeyguide behavior should be predicted by playback amplitude after attenuation in the environment. We measured the mean and maximum amplitudes (in units of A-weighted decibels) of every playback exemplar with a sound-level meter at a distance of 30 m in the natural habitat of these birds

(table S1) and tested whether the amplitudes explained any variance in guiding behavior, either in isolation or in the multivariate models above. In no case did these acoustic measures explain any variance in the probability of being guided or being shown a bees' nest (*7*). Therefore, the honeyguides' elevated response to the honey-hunting sound is unlikely to be explained by its audibility. Instead, the most parsimonious explanation is that honeyguides associate the honey-hunting sound with successful collaboration. Such partner choice should be adaptive by allowing honeyguides to improve their net benefit from interacting with humans.

These results show that a wild animal correctly attaches meaning and responds appropriately to a human signal of recruitment toward cooperative foraging, a behavior previously associated with only domestic animals, such as dogs (*11*). Although humans use many species as foraging partners, including falcons, dogs, and cormorants, these involve trained or domesticated individuals that are specifically taught to cooperate. The honeyguide-human relationship is notable in that it involves free-living wild animals whose interactions with humans have probably evolved through natural selection. To our knowledge, the only comparable relationship involves cooperation between artisanal fishermen and free-living dolphins. Several reports exist of men "calling" dolphins to hunt, starting with Pliny the Elder around 70 CE (*12*). Whether this reflects a similarly specialized communication system to that mediating the honeyguide-human mutualism in Mozambique remains unknown.

How might honeyguides acquire information about honey-hunters' signals of cooperation? Honeyguides are brood-parasitic and reared by insectivorous hosts (*4*), which suggests that their propensity to locate bees' nests and guide humans to them is likely to be innate. However, the "brrrr-hm" human signal studied here is confined to a specific geographical area, and a different cultural group living 1000 km away uses a different signal which is likely to have the same function (*9*). Local adaptation is unlikely to account for corresponding honeyguide specialization, given a lack of obvious genetic structure across its range (*13*). This implies that local refinements to guiding behavior are probably learned, which is supported anecdotally by the belief of many Yao honey-hunters that juvenile honeyguides [which have distinctive yellow plumage (*4*)] are a separate species (called "namandindi") that, despite beckoning humans in the manner of an adult honeyguide ("sego"), falls quiet in response to the honey-hunting sound. We propose that learning might occur socially from conspecifics in the vicinity of bees' nests, resulting in a local cultural tradition among honeyguides that reflects the customs of their human collaborators.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/353/6297/387/suppl/DC1
Materials and Methods
Fig. S1
Table S1
References (*14*–*22*)
Audio S1 to S4

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PROTEIN DESIGN

Accurate design of megadalton-scale two-component icosahedral protein complexes

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Nature provides many examples of self- and co-assembling protein-based molecular machines, including icosahedral protein cages that serve as scaffolds, enzymes, and compartments for essential biochemical reactions and icosahedral virus capsids, which encapsidate and protect viral genomes and mediate entry into host cells. Inspired by these natural materials, we report the computational design and experimental characterization of co-assembling, two-component, 120-subunit icosahedral protein nanostructures with molecular weights (1.8 to 2.8 megadaltons) and dimensions (24 to 40 nanometers in diameter) comparable to those of small viral capsids. Electron microscopy, small-angle x-ray scattering, and x-ray crystallography show that 10 designs spanning three distinct icosahedral architectures form materials closely matching the design models. In vitro assembly of icosahedral complexes from independently purified components occurs rapidly, at rates comparable to those of viral capsids, and enables controlled packaging of molecular cargo through charge complementarity. The ability to design megadalton-scale materials with atomic-level accuracy and controllable assembly opens the door to a new generation of genetically programmable protein-based molecular machines.

The forms and functions of natural protein assemblies have inspired many efforts to engineer self- and co-assembling protein complexes (*1*–*24*). A common feature of these approaches, as well as the structures that inspire them, is symmetry. By repeating a small number of interactions in geometric arrangements that are consistent with the formation of regular structures, symmetry reduces the number of distinct interactions and subunits required to form higher-order assemblies (*2*, *3*, *25*). Symmetric complexes can be designed to form through self-assembly of a single type of protein subunit or co-assembly of two or more distinct types of protein subunits. Multicomponent materials possess several important advantages, including the potential to control the initiation of assembly by mixing independently prepared components. This property could allow,

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SCIENCE

Phonotaxis of Crickets in Flight: Attraction of Male and Female Crickets to Male Calling Songs

Abstract. *Large numbers of two species of mole crickets flew to loudspeakers playing the appropriate calling song outdoors. Mated females were more frequently captured than unmated ones, and males were 12 percent of the catch. Crickets of three other subfamilies were trapped as they flew to mole cricket songs resembling their own.*

Most male crickets and katydids (*1*) make species-specific calling sounds that enable appropriate mute females to find their way to them (*2*). Generally the unmated female walks or runs to the conspecific male. In the only reported case of flight bringing the pairs together, orientation to the conspecific sound occurred before flight (*3*). In studying the acoustic behavior of two species of mole crickets, we discovered that males and mated females as well as unmated females terminated long-range flights (*4*) by orienting to and landing near sources of conspecific calling songs.

Scapteriscus acletus (southern mole cricket) and *S. vicinus* (changa) are important agricultural pests in the southeastern United States. The males burrow in soil and produce calling songs in specially constructed chambers. When we discovered that flying mole crickets were landing near the entrance to the burrow where the male was singing, we broadcast a recorded calling song and dozens of flying mole crickets rained on the speaker. We studied this phenomenon in 1972 and 1973 near Gainesville, Florida, at a lighted golf course and an unlighted pasture. Our experimental setup consisted of two independent broadcast systems and three large metal funnels (1.2 m in diameter) placed 3 to 15 m apart (Fig. 1). Each broadcast system included a battery-operated tape recorder, a battery-operated audioamplifier, and a speaker (*5*). Each speaker was mounted in the center of a funnel and was aimed directly upward. A jar ring was soldered to the bottom of each funnel (5 cm in diameter), and the adults that flew into the funnels were collected in numbered 500-ml jars. The natural calling songs of *S. acletus* and *S. vicinus* were tape-recorded in the field, with the microphone 15 cm above ground level. The soil temperature was 25°C. Synthetic calling songs were made and tape-recorded in the laboratory (*6*). The intensity of the broadcast songs was measured 15 cm above the speaker (*7*) and was maintained at 100 ± 3 db during all experiments (*8*).

Broadcasting trials began about 0.5 hour after sunset and continued until most flight ended, about an hour later. Tests were made only when the soil temperature was 25 ± 3°C. During each trial both calls were broadcast simultaneously, and the trial was ended when at least one of the 500-ml jars contained 20 or more mole crickets. A predetermined duration was not used for each trial because the numbers of mole crickets flying varied greatly at different seasons, dates, and times. Trials in which no jar yielded as many as 20 mole crickets were disregarded. The jars with trapped mole crickets were detached from the funnels after

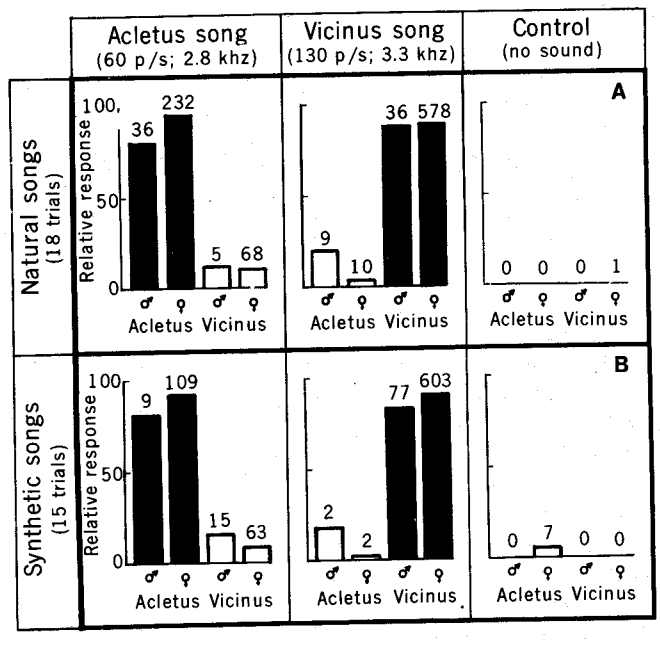

Fig. 1 (left). Sheet metal funnels used to trap mole crickets. The control trap (left back) has no speaker. Fig. 2 (right). Specificity of response of flying *Scapteriscus acletus* and *S. vicinus* to broadcast tape recordings of (A) natural and (B) synthetic calling songs. Crickets captured in traps broadcasting conspecific songs are indicated by black bars. Others are indicated by open bars. Each bar shows the percentage of the total number of a sex and species that was captured by traps during the trials with natural songs or during the trials with synthetic songs. The number of individuals is indicated above each bar; *p/s*, pulses per second.

Table 1. Attraction of other flying crickets to broadcast songs of *Scapteriscus acletus* (60 pulses per second; 2.8 khz).

Subfamily and species	Number attracted		Calling song of attracted species (25°C)		Source of *S. acletus* song
	Male	Female	Pulses per second	Carrier frequency (khz)	
Gryllinae,					
Gryllus rubens	7	12	55	4.8	Natural and synthetic
Oecantinae,					
Oecanthus celerinictus	3	11	65	3.8	Synthetic
Nemobiinae,					
Neonemobius cubensis	0	8	55	7.3	Synthetic

each trial and replaced with empty ones.

The speaker first used to broadcast a particular song was selected randomly; in subsequent trials of the same pair of songs the speakers used were alternated in order to negate position effects. During each trial one of the three funnels served as a control (*9*). At the end of each evening's trials the mole crickets in each numbered jar were identified (*10*), sexed, and counted. For each species, on three or more occasions separated by 1 week or more, 10 to 25 of the trapped females were examined for sperm in the spermathecae.

At the golf course mole crickets could be seen flying at lights 100 m from the funnels. When broadcasting began, the crickets would alter direction and fly toward the funnels. Many dropped or flew into the funnels. Others landed on the sod nearby. At the unlighted pasture the crickets could be seen only as they neared the funnels. Trials involving playbacks of taped natural songs (Fig. 2A) showed that *S. acletus* and *S. vicinus* are principally attracted to their own songs.

Trials with synthetic songs (Fig. 2B) produced results similar to those with natural sounds, thus showing that no differences other than those in the carrier frequency and pulse rate were required to elicit the species-specific responses (*11*). For each species, with both natural and synthetic songs, a larger proportion of females than males showed conspecific responses. A significantly higher proportion (10 percent) of *vicinus* females were trapped at *acletus* songs than vice versa (3.4 percent) ($P < .01$). Of 66 *acletus* females examined, 91 percent had sperm in the spermathecae. Of 56 *vicinus* females, 61 percent had sperm.

Crickets other than mole crickets sometimes flew into the traps broadcasting *acletus* songs (Table 1). In none of the trials were such crickets captured in the silent (control) trap or in the trap broadcasting *vicinus* songs. Although the three species captured represented three subfamilies, their calling songs were similar to that of *acletus*. Specifically, the calling songs of all three were trills with pulse rates at 25°C within five pulses per second of the *acletus* trill. Males of only two of the species were captured. At least some of the *Gryllus rubens* females had mated before capture, for they subsequently laid fertile eggs.

The attraction of virgin female crickets to male calling songs is easily understood. The attraction of females with sperm in their spermathecae (*12*) and especially the attraction of males are less easily interpreted. We interpret the flights of these individuals (and perhaps of virgin females, too) as dispersive, and we suggest that they are using the sexual signaling of males of their species as an indication of a habitat suitable for colonization. We originally thought that those flying toward the sound as a habitat-indicating signal and those flying toward it as a male-indicating signal should end their flights differently. We therefore predicted that those landing within the sound trap would include a significantly higher proportion of females than those landing outside the trap. However, we compared the sex ratio of 769 *acletus* that had landed outside the trap with that of 694 that had landed inside and found no significant difference ($P = .21$). We now believe that both sexes, and mated as well as unmated females, home in on the sound with equal accuracy, but we conjecture that on landing the virgin females run to the singing male's burrow while others run and burrow elsewhere. Morris (*13*) demonstrated phonotaxis by males to male calling songs in conocephaline katydids; however, the context was male-male aggression over occupation of territory (or broadcasting space). Male-male aggression occurs in crickets and has been studied and described, but approach of males to male calling songs has not been reported (*14*). We have never observed it in earthbound mole crickets.

Attraction of large numbers of flying crickets may prove useful in control—either as a means of destroying crickets or as a means of timing control procedures. We have already put it to use in studying the flight ranges of mole crickets and in studying the features of the songs responsible for species-specific responses (*4, 11*).

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5. We used Nagra III and Nagra IV tape recorders during 1972 and cassette tape recorders (Bell & Howell model 294, 7235, and Sony model TC-66) during 1973. The latter were checked daily for excessive flutter and aberrant tape speed. The amplifiers were an Alton Electronic audio amplifier (1972) and a Realistic model MPA-20 (1973). The speakers were Realistic model 40-1228 covered with aluminum wire screen.
6. The electronic synthesis was similar to that described by T. J. Walker, *Ann. Entomol. Soc. Am.* **50**, 629 (1957). The pulse interval ratio was maintained at 1 : 1.
7. A General Radio model 1551-B sound level meter was used to monitor the intensity.
8. The intensity of calling males measured in the field was 42 to 92 db at 15 cm above the burrow entrance. We discovered that the catch of mole crickets in sound traps was dramatically increased by using higher than natural sound levels.
9. An empty funnel with no sound (control) generally caught no mole crickets. It never had more than 1.5 percent of the number of mole crickets in the other two traps.
10. *Scapteriscus* species can be easily identified by morphological characters [W. S. Blatchley, *Orthoptera of Northeastern America* (Nature Publishing, Indianapolis, Ind., 1920)].
11. Further experiments with synthetic sounds showed that the species-specific response of *S. acletus* includes discrimination of both pulse rate and carrier frequency (S. M. Ulagaraj and T. J. Walker, in preparation).
12. One of us (S.M.U.) has twice observed a female land and enter the burrow of a calling male. One of the two females was dug out a few minutes later and found to have sperm in the spermathecae. It is doubtful that mating was completed during the interval. We do not know whether female mole crickets mate more than once or with more than one male. Because they lay substantially fewer (but larger) eggs than most other female crickets, we doubt that multiple matings are usual.
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Report

Social Transmission of Novel Foraging Behavior in Bats: Frog Calls and Their Referents

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Summary

The fringe-lipped bat, *Trachops cirrhosus*, uses prey-emitted acoustic cues (frog calls) to assess prey palatability [1]. Previous experiments show that wild *T. cirrhosus* brought into the laboratory are flexible in their ability to reverse the associations they form between prey cues and prey quality [2]. Here we asked how this flexibility can be achieved in nature. We quantified the rate at which bats learned to associate the calls of a poisonous toad species with palatable prey by placing bats in three groups: (a) social learning, in which a bat inexperienced with the novel association was allowed to observe an experienced bat; (b) social facilitation, in which two inexperienced bats were presented with the experimental task together; and (c) trial-and-error, in which a single inexperienced bat was presented with the experimental task alone. In the social-learning group, bats rapidly acquired the novel association in an average of 5.3 trials. In the social-facilitation and trial-and-error groups, most bats did not approach the call of the poisonous species after 100 trials. Thus, once acquired, novel associations between prey cue and prey quality could spread rapidly through the bat population by cultural transmission. This is the first case to document predator social learning of an acoustic prey cue.

Results and Discussion

Social learning can expand the foraging repertoire of a given individual and enhance predator foraging success [3–5]. Numerous studies have demonstrated the ability of predators to learn socially about prey cues in the olfactory and visual modalities (see [5] for review). Predator social learning of acoustic prey cues, however, has not been documented. Here we test the ability of the predatory bat, *T. cirrhosus*, to acquire a novel acoustic association for prey via social learning.

T. cirrhosus has a unique ability among bats to prey on frogs by listening to the advertisement calls male frogs produce to attract their mates [1]. In a previous study, we investigated the associations *T. cirrhosus* forms between prey cues (species-specific prey mating call) and prey palatability [2]. Using a fading-conditioning paradigm [6], we were able to rapidly reverse the bats' assessment of palatable and poisonous prey.

Here we ask whether this flexibility is part of the bats' natural foraging repertoire and to what degree novel associations between prey cue and prey quality can be culturally transmitted. To address these questions, we quantified the rate of acquisition of a novel foraging behavior in three learning groups: (a) a social-learning group, (b) a social-facilitation group, and (c) a trial-and-error group. The target foraging behavior was the bats' ability to learn to associate the calls of the sympatric cane toad, *Bufo marinus*, with a palatable food reward. *B. marinus* is both highly poisonous and far too large for a *T. cirrhosus* to eat, so on two accounts it should be an unsuitable prey item. The criterion for task acquisition was flying to and landing on a speaker broadcasting toad calls in three consecutive trials.

We first conducted baseline tests with all bats to determine initial responses to *B. marinus* calls. None of the bats showed any initial response to *B. marinus* calls. We then tested for social learning by allowing an inexperienced bat to observe the foraging behavior of an experienced bat (tutor) that had already acquired the novel association. The first tutor learned to associate toad calls with a palatable food reward via a fading-conditioning technique (for methods, see [2]). Subsequent tutors acquired the association via social learning, such that the test bat in one experiment became the tutor in the next experiment.

The novel foraging association was transmitted successfully among all bats in the social-learning group ($n = 10$). There were two series of sequential interactions, or "chains" (Figure 1). The first chain consisted of two social-learning transmissions among three bats. The third bat died in captivity, and so the fourth bat was conditioned via fading, initiating a second chain of eight social-transmission events among nine bats, at which point we halted the experiment. Bats in the social-learning group acquired the novel foraging task in 5.3 ± 1.7 trials (mean ± SEM, range = 1–11 trials, Figure 2). There was no degradation in the rate of acquisition from bat to bat (Pearson product-moment correlation: $r = 0.314$, $p = 0.377$; Figure 1).

To control for possible motivational effects associated with the mere presence of a second bat [7] in the social-facilitation group, we quantified the rate of acquisition of a test bat housed with an inexperienced conspecific ($n = 5$). In addition, in the trial-and-error group we quantified the rate of acquisition of bats housed alone, when the only possibility for task acquisition was individual learning ($n = 5$). Rates of task acquisition were significantly slower for these two groups in comparison with the social-learning group (Kruskal Wallis test: $H = 15.28$, $p < 0.001$, Figure 2). There was no significant difference in the rate of acquisition between the social-facilitation group and the trial-and-error group (mean trials to acquisition ± SEM: 96.8 ± 3.2 and

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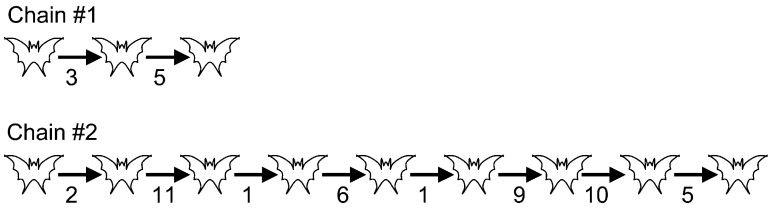

Figure 1. Chains of Transfer in the Social Learning Group

Arrows indicate the transmission of the novel foraging behavior; the numbers underneath the arrows indicate the number of trials required to reach acquisition criterion for each bat. After testing, the current test bat became the tutor for the next test bat.

96.2 ± 3.8, respectively. Mann-Whitney U test: U = 12.0, p = 0.881, Figure 2). If a bat never acquired the novel foraging task, we assigned it an acquisition score of 100 trials; thus, results are conservative measures of task acquisition.

In addition to demonstrating a profound potential for cultural transmission of acoustic-cue associations used in foraging, our results provide evidence as to how such associations might originate in the wild. Most of the bats in the trial-and-error group and the social-facilitation group never learned the novel association between prey cue and prey quality. In each of these groups, one of five bats learned the novel association after more than 80 trials (in the social-facilitation group, this individual reached criterion at 84 trials, and in the trial-and-error group it did so at 81 trials). Thus, although it's rare, bats will inspect calls of poisonous species on their own. This exploratory behavior could enable bats to encounter and track novel sources of prey and could account for the origin of novel associations between prey cue and prey quality in the wild. Such behavior could thus provide a starting point for the rapid cultural transmission that we show here for foraging information among bats.

In other taxa it has been suggested that if mistakes are not costly, it is advantageous for animals to periodically sample their environment. Buchler [8] suggested that wandering shrews (*Sorex vagrans*) make "intentional errors," even when they know where profitable food patches are located, in order to update their knowledge of their surroundings. Likewise, great tits (*Parus major*) have been shown to sample a variety of prey species, even when they have a profitable search image for an abundant prey type [9]. In lowland Neotropical rainforests, there can be extreme fluctuations in the abundance of available prey [10]. In the rainy season, frogs call conspicuously, but in the dry season, frogs are harder to find, and *T. cirrhosus* likely has to rely more heavily on non-anuran prey. It is possible that *T. cirrhosus* uses exploratory behavior in combination with social learning to track local and seasonal changes in prey abundance. Because *T. cirrhosus* uses many sensory modalities to assess its prey (e.g., prey-emitted acoustic cues, echolocation cues, and chemical cues), it is likely that a mistake at one level of prey assessment would be corrected at another level. As such, mistakes should not be costly, and behavioral flexibility should be advantageous.

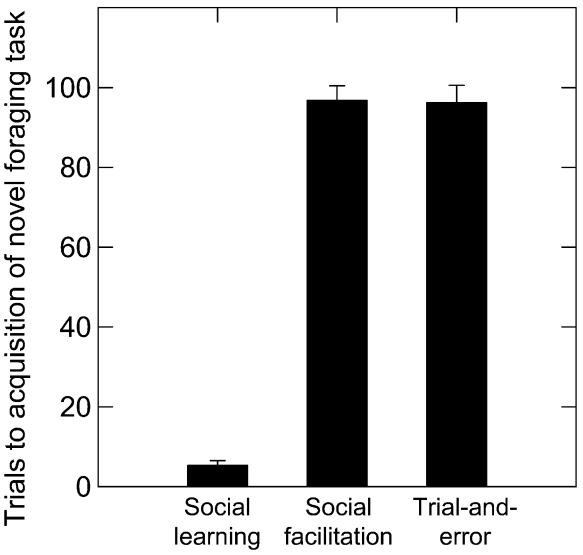

Figure 2. Mean Rates of Acquisition, ± SEM, of the Novel Foraging Task for the Three Learning Groups

Johnston and Fenton [11] found that pallid bats (*Antrozous pallidus*) vary tremendously in their feeding habits, both between and within populations. Indeed, variation seems to be the rule in many species of bat (e.g., [12, 13]). Dietary studies have shown that *T. cirrhosus* preys on a wide variety of prey items, including frogs, insects, fruit, smaller species of bats, and even birds [14–16], with insects composing the largest component of the diet. No seasonal differences in prey consumption were found in analysis of *T. cirrhosus* stomach contents in Brazil, although this could be due to differences in breeding phenology of frogs present in the areas sampled [14]. Further investigation is necessary for determining whether dietary patterns and foraging preferences vary with season and whether this can be linked to learning within social groups.

Our results clearly demonstrate that if one bat forms a novel acoustic association, in this case evaluating a toad call as a signal of palatable prey, then the association can spread rapidly from bat to bat through cultural transmission. All that is required is that bats observe one another feeding in nature.

T. cirrhosus bats are social. They roost in groups of four to 50 or more individuals [17]. Multiple bats can forage simultaneously at a frog chorus [1, 18], and individuals are often captured together in the same mist net at a foraging site (R.A.P. and M.J.R., unpublished data). Thus, in addition to roosting together, *T. cirrhosus* individuals are known to congregate at feeding sites, thus enabling the observation of foraging conspecifics.

Many species of bat have social structures and foraging habits that should facilitate social learning [19]. Bats are long-lived animals, they tend to form stable groups, and they often feed on temporally and spatially fluctuating resources; thus, cultural transmission of foraging information should increase foraging success [19, 20]. Several studies have shown that bats attend to the foraging behaviors of conspecifics. Many species of

insectivorous bats produce loud echolocation calls with distinctive terminal phases as they approach and capture prey. Other individuals eavesdrop on these conspicuous signals and use them to detect aggregations of prey [21, 22]. Likewise, the screech calls of greater spear-nosed bats, *Phyllostomus hastatus*, attract group mates to foraging patches, such as concentrations of flowering balsa [23]. Studies with short-tailed fruit bats, *Carollia perspicillata*, suggest that social learning about temporally fluctuating food resources may even take place at the roost; bats have been shown to use olfactory cues associated with returning roost mates to shape their foraging preferences [20]. These factors suggest that social learning may be common in bats; however, only a handful of studies have demonstrated that bats indeed learn socially about foraging [20, 24–26].

Recent studies have investigated the role of matrilineal kin groups in bat roosting and foraging behavior. Although Kerth et al. [27] found evidence for information transfer about roost sites in Bechstein's bats, *Myotis bechsteinii*, they found no evidence for information transfer about feeding sites. In both the Kerth et al. study and a study of greater horseshoe bats (*Rhinolophus ferrumequinum*), however, radiotracking data demonstrate that mothers and their daughters shared foraging grounds, sometimes for years [27, 28]. Thus, the vertical transfer of foraging-site location from mother to pup could be playing a large role in the foraging dynamics of these bat communities. Although the learning we document in our study is likely entirely opportunistic (the result of one bat eavesdropping on the successful foraging behavior of another), the study of social learning in highly related groups, and especially in mother-pup pairs, should prove an interesting area for further research.

Our study is not designed to distinguish among the mechanisms of social learning [29–32]; however, it is likely that these bats are learning by either stimulus enhancement or observational conditioning. In stimulus enhancement the activity of the tutor draws the observer's attention to the test stimulus [31, 33]—in our experiment, to the toad calls. The observer then forms an association between the stimulus and the reward via individual, trial-and-error learning. Because we altered the speaker location for each trial, we can rule out the possibility that the bats are learning to associate a food reward with a particular spatial location (local enhancement).

In observational conditioning, a type of higher-order conditioning, the observer associates the stimulus with the outcome experienced by the tutor and thus responds more readily to the stimulus itself [31, 34]. In our social-learning treatment, the test bat did not initially attend to the toad calls or to the flight of the tutor bat. In the initial trials, the test bat typically would commence responding with ear motions and head orientation only once the tutor began to consume the food reward, and only in later trials did the test bat respond to the test stimulus. These observations suggest that the sensory cues associated with food consumption by a nearby bat may serve to trigger attention and thus expedite the acquisition of novel foraging associations.

Once the attention of the test bat was elicited, a number of sensory cues could have been involved in the test bat's observation of the tutor. Our experiments were conducted under low-light conditions, such that visual observation was possible. As *T. cirrhosus* approaches a target, the rate of its echolocation calls increases [35]; thus, the test bat could have used the patterns of the tutor's echolocation calls to follow its behavior. It could also have tracked the movements of the tutor bat with its own echolocation calls, and it could have passively listened to the crashing noise produced when the tutor bat landed on the screen. Further investigation is necessary to determine both the learning mechanisms and the sensory cues involved in this social interaction.

The flexibility, exploratory behavior, and social learning we document in this study endow the bats with the potential to respond rapidly to changes in prey conditions. With the catastrophic and worldwide decline of amphibians [36–38], their predators' ability to track such changes becomes increasingly critical. Rather than falling into ecological traps, unable to quickly alter previously adaptive behavior [39], *T. cirrhosus* should be able to respond quickly to changes in the prey community, the extinction of preferred prey items, and the introduction of novel prey species.

Experimental Procedures

Experiments were conducted at the Smithsonian Tropical Research Institute field station on Barro Colorado Island (BCI), Panama, from February to June 2004 and 2005. We captured the bats in mist nets and tested them in a 4.5 m × 4.5 m × 2.5 m outdoor flight cage. We illuminated the flight cage with a 25 watt red light bulb to facilitate our observations of the bats. This light level was within the range of illuminations in which the bats forage. We used a Sony NightShot DCR-TRV340 camera equipped with a Sony HVL-IRH2 infrared light to record all initial and final tests, all social learning trials, and a subset of the social-facilitation and trial-and-error learning trials. Each bat was marked with a passive integrated transponder (PIT tag) and released at its site of capture after testing. All experiments were licensed by the Smithsonian Tropical Research Institute and the University of Texas at Austin (IACUC #04113002).

Stimulus Presentation

We broadcast calls of *B. marinus* from a Dell Inspiron 8100 computer, a SA-150 Realistic amplifier, and 40-1040 Radio Shack speakers. To approximate the natural call intensity of *B. marinus* in the wild, we broadcast the calls at an amplitude of 75 dB SPL (re. 20 μP) measured at a distance of 1 m from the speaker. Most of the energy in *B. marinus* calls falls between 548 and 708 Hz; the frequency response of these speakers is flat for these frequencies. To ensure that the bats responded to the acoustic stimulus broadcast and not to the speaker itself, we concealed one to five speakers beneath a 1.5 m × 1.5 m screen covered with leaf litter and randomly repositioned the speakers between trials. To ensure that the bats were responding to the toad calls per se and not to other noises associated with the speaker, in a subset of the trials we turned on one of the control speakers and broadcast a sound file of silence. The bats never approached control speakers. Toad calls were broadcast for 60 s or until the test bat landed on the speaker, whichever came first. Trials were conducted in approximately 10 to 15 min intervals with a maximum of 20 trials per night.

Food Rewards and Motivation Levels

Because frogs are protected on BCI, small bait fish were purchased, frozen, thawed, and used as food rewards for the learning trials. The bats readily consumed the fish. To ensure that the bats were not responding to extraneous cues associated with the rewards, in all learning trials we placed multiple rewards in random locations on the screen. Bats only approached food rewards placed on active speakers.

T. cirrhosus picks up its prey in its mouth and flies with it to a nearby perch to consume it. *T. cirrhosus* generally carries only one prey item at a time. We placed many pieces of fish on the active speaker so that if both the tutor and the test bat landed on the speaker in a given trial, each bat would receive a food reward. We closely monitored the bats' food intake to ensure high motivation levels, and trials were conducted only when bats were motivated to feed.

Between trials, we periodically broadcast a probe stimulus known to elicit response in *T. cirrhosus* (the calls of a preferred prey species: either a complex túngara frog call or a chorus of túngara frog calls [1, 40]). If the test bat did not respond to the probe stimulus, we could infer that a lack of response to the toad calls could have been due to factors other than the salience of the test stimuli (i.e., handling stress, fear of new environment, satiation). Bats always responded readily to the probe stimuli in all probe tests.

Summary of Trials

1. Initial Trials

To determine its baseline response to toad calls, we first tested each bat alone, without rewards on the speaker. Toad calls were broadcast for 60 s or until the bat approached and landed on the speaker, whichever came first. Three initial tests were conducted for each bat.

2. Learning Trials

Three types of learning trials were conducted: (a) social learning, (b) social facilitation, and (c) trial and error. Learning trials were conducted until criterion (flying to and landing on the speaker in three consecutive trials) had been reached or until 100 trials had been conducted, whichever came first. Food rewards were placed on the speaker in all learning trials.

3. Final Trials

After learning trials, we removed the tutor bat and conducted three final trials with the test bat alone. The protocol for final tests was identical to initial tests. Final test results mirrored criterion results: all bats that reached criterion in the learning trials responded to toad calls by flying to and landing on the speaker in all three final trials. No bat that failed to reach criterion in the learning trials responded in the final trials.

Supplemental Data

One supplemental figure is available with this article online at http://www.current-biology.com/cgi/content/full/16/12/1201/DC1/.

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RECOGNITION AND SELECTION OF SETTLEMENT SUBSTRATA DETERMINE POST-SETTLEMENT SURVIVAL IN CORALS

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Abstract. Habitat recognition and selective settlement by dispersive propagules greatly increases the post-settlement survival chances of sessile organisms. To better understand the key role some species can play in the structure of highly complex coral reef ecosystems, we compare the role of two independent, but sequential, processes: settlement choice and post-settlement survival. This study describes the chemical and physical recognition and ranking of specific settlement substrata by coral larvae. Several species of crustose coralline algae (CCA) are known to induce coral settlement; however they also employ physical and biological anti-settlement defense strategies that vary greatly in effectiveness. We examine the interactions between settling larvae of two common reef building coral species (*Acropora tenuis* and *A. millepora*) and five species of CCA (*Neogoniolithon fosliei*, *Porolithon onkodes*, *Hydrolithon reinboldii*, *Titanoderma prototypum*, and *Lithoporella melobesioides*) that co-occur on reef crests and slopes of the Great Barrier Reef, Australia. Distinct settlement patterns were observed when coral larvae were provided with a choice of settlement substrata. Settlement on the most preferred substratum, the CCA species *T. prototypum*, was 15 times higher than on *N. fosliei*, the least preferred substratum. The rates of post-settlement survival of the corals also varied between CCA species in response to their anti-settlement strategies (shedding of surface cell layers, overgrowth, and potential chemical deterrents). Rates of larval settlement, post-settlement survival, and the sensitivity of larvae to chemical extracts of CCA were all positively correlated across the five species of CCA. Nonliving settlement substrata on coral reefs is sparse; consequently the fact that only a few CCA species (notably *T. prototypum*) facilitate coral recruitment, has important implications for structuring the reef ecosystem.

Key words: coral; coralline algae; defense; Great Barrier Reef, Australia; metamorphosis; recruitment; settlement; shedding; substrata; survival.

INTRODUCTION

The distribution and abundance of sessile organisms is often governed by the settlement of their mobile propagules. For marine organisms, larval settlement and early post-settlement survival can explain much of the variance in adult populations (Raimondi 1990, Palma et al. 1999). Settlement strategies of mobile larvae vary from passive, indiscriminate settlement to active substratum selection, which can help minimize the chances of settling in unsuitable habitats (e.g., Grosberg 1981). One of the recurrent themes of marine ecology has been the tendency to underestimate the capacity of larvae to influence their fate (Young and Chai 1985); however recent research suggests habitat selection by larvae at settlement has a strong influence on post-settlement survival and adult distribution patterns (Mundy and Babcock 1998, Baird et al. 2003).

Coral reefs are highly diverse marine communities constructed and dominated by sessile organisms that disperse via a planktonic larval stage in their early life history. Microhabitats on coral reefs conductive to coral larval settlement and persistence are limited. Unoccupied primary substrata are rare and most other surfaces such as live corals, filamentous turf algae, and sediment are poor habitats for settlement and survival. Coral planula choose their site of permanent attachment based upon physical factors such as light, salinity, water motion, depth, surface orientation, and sedimentation (Maida et al. 1994, Mundy and Babcock 1998, Raimondi and Morse 2000). Living surfaces of nongeniculate or ''crustose coralline'' algae (CCA; Rhodophyta, Corallinaceae) can aid in the survival of settlers by excluding other space competitors and providing protection from sediment entrapped in turf algae (Babcock and Mundy 1996, Ruiz-Zarate et al. 2000). The suitability of a substratum as attachment site is however primarily determined by chemical and/or biological surface properties, such as surface films of algae, diatoms, or bacteria (Morse et al. 1988, Johnson et al. 1997, Raimondi and Morse 2000, Baird and Morse 2004).

Settlement and metamorphosis in many scleractinian corals is induced by external biochemicals (morphogens) associated with living CCA (Morse et al. 1988,

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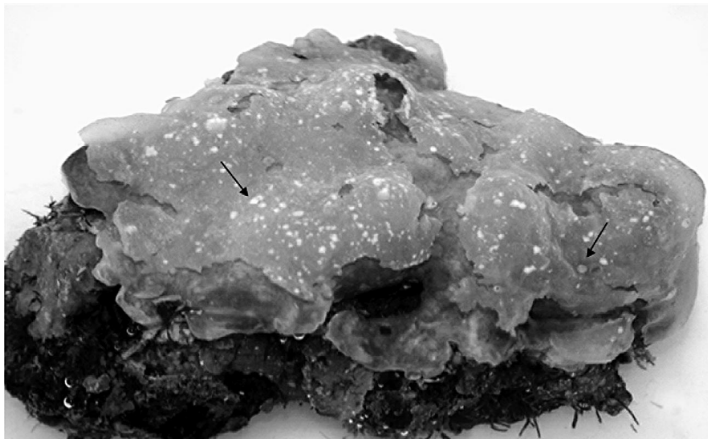

PLATE 1. Example of an anti-settlement defense mechanism in crustose coralline algae (CCA): epithallial shedding of >80% of thallus surface, removing most of the two-day-old coral settlers on the CCA species *Neogoniolithon fosliei* in a single sloughing event. Two of the settlers are marked by arrows. Scale: ~1:1. Photo credit: L. Harrington.

1996, Heyward and Negri 1999). These morphogens were identified as cell wall bound polysaccharides (Morse and Morse 1991). Although many species of CCA are capable of inducing metamorphosis in laboratory experiments, each species has specific characteristics that could influence its ecological relevance to habitat selection by the larvae. Larvae of the brooding Caribbean coral *Agaricia humilis* exhibited species-specific settlement preferences among five unidentified CCA species in laboratory experiments (Morse et al. 1988). Later research showed that larval settlement in this species was associated with the common CCA *Hydrolithon boergensii* (Raimondi and Morse 2000). A recent field study on the Australian Great Barrier Reef (GBR), has demonstrated a species-specific preference of broadcast-spawning corals to recruit on the coralline alga *Titanoderma prototypum* (R. Steneck, E. Turak, L. Harrington, and T. Done *unpublished manuscript*). The field observation of a high abundance of coral recruits associated with *T. prototypum* suggests this species may play a critical role in facilitating coral recruitment on the GBR.

Coral larvae may be induced to settle by properties of the substrata; however, subsequent mortality of recruits, caused by active substratum defenses, may result in the high rates of juvenile mortality. The potential for CCA to play a negative role in coral recruitment by increasing post-settlement mortality and reducing growth rates of coral recruits has not been considered. Many species of CCA eliminate newly settled organisms by shedding epithallial cells, i.e., sloughing (e.g., Keats et al. 1997). Overgrowth is also an important strategy of interference in the competition between space-limited organisms (Sebens 1986, Airoldi 2000). Thick CCA can easily overgrow newly settled corals (Maida et al. 1994, Dunstan and Johnson 1998), and even established adult corals (Antonius 2001). Chemical defense, in particular allelopathy, is a third widespread antifouling strategy used by many sessile organisms (Maida et al. 1995, Suzuki et al. 1998). Although chemical cues are known to induce settlement and metamorphosis of coral larvae on CCA, the potentially allelopathic nature of some of their metabolites have so far received little attention.

To better understand the key function some CCA species can play in the structure of coral reef ecosystems, we have examined the role and relationship of two independent but sequential processes: selective settlement and post-settlement survival of coral larvae. We quantified the settlement preferences of larvae of the two reef building corals *Acropora millepora* and *A. tenuis* on horizontal and vertical surfaces of living and dead CCA species, and on two inert substrata. We then quantified survivorship of newly settled corals on these substrata to determine if species-specific habitat selection influences post-settlement mortality levels. We also examined the chemically mediated induction of coral settlement by different species of CCA, and assessed the relationship of this process with that of selective settlement and post-settlement survival.

METHODS

We examined how both physical and chemical attributes of CCA influence larval settlement and metamorphosis (Experiment 1a and b) and post-settlement survival of juvenile corals (Experiment 2) in a controlled laboratory environment. We also examined the presence of chemical inducers for metamorphosis (morphogens) in each species of CCA (Experiment 3). Experiments were conducted with two species of the coral genus *Acropora*, representing the dominant genus on the Great Barrier Reef (GBR) collected from three spawning events, and five species of common CCA found on offshore reefs of the GBR. Settlement experiments were performed in outdoor aquaria at the Australian Institute of Marine Science (AIMS, Townsville, Queensland), and at the Lizard Island Research Station (LIRS, Great Barrier Reef, Queensland).

Obtaining coral larvae

Experiments 1a and 2.—Ten mature colonies of *Acropora tenuis* were collected prior to spawning from

Nelly Bay, Magnetic Island (19°10′ S, 46°51′ E). Spawning occurred on 2 November 2001.

Experiment 1b.—Six mature colonies of *Acropora millepora* were collected from Lizard Island (14°40′ S, 145°26′ E). Spawning occurred on 21 November 2002.

Experiment 3.—Six mature colonies of *A. millepora* (Ehrenberg 1834) were obtained from the Whitsunday Islands (20°15′ S, 148°50′ E), which spawned at AIMS on 5 December 2001.

For all experiments the gametes were fertilized and larvae cultured as per Heyward and Negri (1999).

Collection of non-geniculate crustose coralline algae

Experiments 1a, 2, and 3.—CCA were collected from the slopes of Davies Reef (18°50′ S, 147°742′ E) at 3–7 m depth. Five common species of CCA were collected on scuba using hammer and chisel: *Hydrolithon reinboldii*, *Neogoniolithon fosliei* (see Plate 1), *Porolithon onkodes*, *Lithoporella melobesioides*, and *Titanoderma prototypum*. The identity of each specimen was verified under a dissecting microscope using reproductive and vegetative morphological and anatomical features as diagnostic characters (Gordon et al. 1976, Adey et al. 1982). The epithallial surface area of each fragment was determined using the foil wrap technique (Marsh 1970; median area: 16 ± 0.9 cm^2, mean ± 1 SE). CCA fragments were maintained at AIMS in an outdoor 1000-L flow-through tank under a pivoted PVC trough that tipped ~25 L of seawater every ~2 min to mimic a high-energy reef environment. One week after collection, the fragments were cleaned to remove all epibionts prior to experimental manipulation.

Experiment 1b.—The same CCA species (except for *L. melobesioides*) were collected at Lizard Island from the reef crest and slope between 3 and 7 m depths, and maintained in outdoor 25-L flow-through tanks at LIRS. Total thallus surface area averaged 11 ± 0.4 cm^2.

Substratum-specific settlement rates

Experiment 1a: Acropora tenuis *larvae.*—This experiment was performed to determine the effects of substratum type, the health of the CCA, and substratum orientation on settlement rates of *A. tenuis* larvae. Half of the CCA fragments of each species were kept alive, while the other half were killed by rinsing in fresh water for one hour and sun drying for five hours. Two additional treatments of inert substrata were prepared by cutting similarly sized fragments from unglazed five-day conditioned terracotta and from dead skeleton of the massive scleractinian coral *Porites* sp. One-half of the live and dead fragments were embedded onto glass slides using underwater epoxy putty (AquaStik, Aquasonic, Ingelburn, New South Wales, Australia) allowing for vertical deployment. One replicate of each treatment (both live and dead vertical and horizontal fragments of five CCA species and two inert substrata) was placed in each of five aerated aquaria containing unfiltered seawater. These aquaria were placed within a large outdoor flow-through tank, acting as a water bath, under 90% shading to minimize overgrowth by filamentous algae. Nine days after fertilization, when the majority of larvae in the culture were competent to settle, ~8000 larvae were added to each of the five aquaria, where they were simultaneously presented with all substrata treatments. Three days after larval transfer, when many of the larvae had either settled or ceased swimming, the numbers of metamorphosed corals were determined on each fragment, using the criteria of Heyward and Negri (1999).

Experiment 1b: Acropora millepora *larvae at LIRS.*—To create stable horizontal settlement surfaces, all CCA fragments were embedded into plastic petri dishes with nontoxic underwater epoxy (Z-spar Splash Zone Compound, Kop-Coat, Pittsburgh, Pennsylvania, USA). Sixteen replicates of each of the CCA species, and fragments of terracotta tiles of similar sizes, were placed into eight replicate aquaria containing unfiltered seawater. At five days of age, ~3000 larvae were added to each of the aquaria.

Post-settlement survival

Experiment 2: Survival of Acropora tenuis *spat in the laboratory.*—In order to assess early post-settlement survivorship, the number of *A. tenuis* spat needed to be increased on all substrata except for *T. prototypum*. To achieve this, all *T. prototypum* fragments were removed from the aquaria after three days of exposure to larvae. Within three more days, the number of settled and metamorphosed spat on all remaining substrata had increased by 19% (an additional 400 spat). After a total of six days exposure to larvae, all CCA fragments were transferred from the aquaria to perforated plastic trays and placed within the outdoor flow-through 1000-L tank with unfiltered seawater and the water-dumping PVC trough to remove sloughed cell layers. Survivorship of *A. tenuis* on the fragments was examined under a dissecting microscope daily for the first 30 days, and once more 6 months later.

Investigating chemical responses

Experiment 3: Larval metamorphosis in response to CCA morphogen concentrations.—We examined whether each CCA species contained extractable chemical inducers to trigger metamorphosis (morphogens) in the coral larvae tested. Sterile extracts from each of the CCA species, terracotta, and *Porites* sp. were prepared by individually grinding a known wet mass of chips of each substratum in methanol (HPLC grade). The slurry was passed through 0.45-μm, methanol washed nylon filters (47 mm diameter, Gelman Science, Ann Arbor, Michigan, USA) and the filtrate retained. The extraction process was repeated and the filtrates combined and evaporated to dryness under N_2. The extracted residues were then resuspended in 0.2-μm filtered seawater to a stock concentration equivalent to

500 mg CCA/mL and stored frozen. Control extracts were prepared as described but with addition of either ground *Porites* or terracotta, or without the addition of ground substrata.

To test for settlement competency, larvae were exposed each day to 5 × 5 mm chips of *P. onkodes* (Heyward and Negri 1999). High levels of metamorphosis (>70%) were achieved after six days in 2001 and four days in 2002; hence the CCA extract induction experiments were performed with seven- and five-day-old larvae, respectively.

Larval metamorphosis assays were performed in sterile 10-mL wells (six-well culture plates, Nunc, Hongo, Bankyo-ku, Tokyo, Japan) maintained in a constant temperature room at 120 μmol quanta·m^{-2}·s^{-1} for 12 h/d, and strong fan-forced air flow to maximize gas exchange on the seawater surface in the wells. The temperature was set to 28–29°C in 2001 and 26–27°C in 2002. Coral larvae (n = 10–20) were introduced to each well containing 0.2-μm filtered seawater and the CCA extract to a final volume of 10 mL. The concentration range of CCA extract added to the wells was 0–50 mg CCA/mL seawater, with the concentration based upon the original wet mass of CCA extracted to enable comparison between species. For the highest extract concentration (50 mg/mL), the mass of organics added was ~150 μg total extract/mL. Six replicate wells were used for each treatment. Early-stage settlement and metamorphosis was assessed after 36 h using a dissecting microscope.

Statistical analyses

Experiment 1a and b.—Log-linear models were used to analyze larval settlement rate as a function of settlement substratum (five CCA species and two controls), status of CCA fragment (live and dead), orientation (horizontal and vertical), and sampling year. The initial model involved main effects and all interactions. The final model was selected by backward elimination of nonsignificant terms ($P > 0.05$). Overdispersion was present in the data (McCullagh and Nelder 1989), and F ratio tests based on the mean square deviance were used to assess the significance of effects.

Experiment 2.—For the survival data, we investigated how the instantaneous probability of larval survival at a given point in time (the hazard) varied with the treatments. The use of parametric and Cox proportional hazards models were assessed; however the model assumptions could not be satisfied for either approach. Since all censoring (discontinuation of observations on surviving larvae) occurred at the end of the experiment, it was possible to analyze the hazard for each period without the censoring resulting in biased estimates. The response variable was the number of deaths during a period divided by the number alive at the beginning of the period. Log-linear models were used with the hazard as the response, and the explanatory variables were settlement substratum, orientation, day, and aquaria nested in species. The variation due to aquaria was used as the error term for effects not involving day, and the mean deviance was used to assess all other effects (McCullagh and Nelder 1989). Partial effects plots were used to show the results; these plots show the effect(s) of one variable in a model adjusted for the effects of all others.

Experiment 3.—To investigate chemical induction of metamorphosis, the proportions of metamorphosed coral larvae were modeled using generalized additive models (Hastie and Tibshirani 1990) for the five species of CCA. Since the responses were proportions that varied smoothly with dose, we used binomial models with smoothing splines. The dose level at which the maximum response was observed was estimated from the fitted response curves. Confidence intervals for the maximum response dose for each species were obtained by taking bootstrap samples of the data (N = 1000), refitting the models, calculating the maximum response dose for each bootstrap sample, and taking the median as an estimate of maximum responses and 10th and 90th percentiles of the distributions as the end points of 90% confidence intervals (Davison and Hinkley 1997). Differences between the species were assessed using permutation tests (N = 1000).

Synthesis.—The properties of the five species of CCA used in the three experiments were related to each other by examining correlations between the species effects across the three pairs of experiments.

Results

Experiment 1a and b: Substratum-specific settlement rates of Acropora tenuis *and* A. millepora *larvae.*—Nine-day-old *Acropora tenuis* and five-day-old *A. millepora* larvae were observed to settle on all substrata tested, regardless of species, orientation, and health status, but the number of settling larvae varied greatly between the different treatments (Fig. 1). Log-linear analyses of the coral settlement rates onto CCA substrata revealed all interactions to be nonsignificant ($P > 0.05$). Differences in rates of settlement between the two sampling years were also nonsignificant, while all other main effects were significant: species ($F_{7,77}$ = 7.12, $P < 0.001$), orientation ($F_{1,77}$ = 13.5, $P < 0.001$), and status ($F_{1,77}$ = 41.0, $P < 0.001$). Settlement varied greatly across the five species (Fig. 1), being 14.9 times higher (95% CI = (5.5, 40.5)) on *T. prototypum* (the best settlement inducer) than *N. fosliei* (the weakest settlement inducer). In all CCA species, living fragments induced greater settlement and metamorphosis than dead fragments, settlement being 9.4 (3.7, 23.6) times higher for live rather than dead substrata. On all substrata, settlement on horizontal surfaces was 2.8 (1.5, 5.1) times higher than on vertical surfaces.

Experiment 2: Survival of Acropora tenuis *spat.*—Survival experiments revealed a very similar pattern to that observed for settlement. The variation in survival of coral spat between substrata was extreme (Fig.

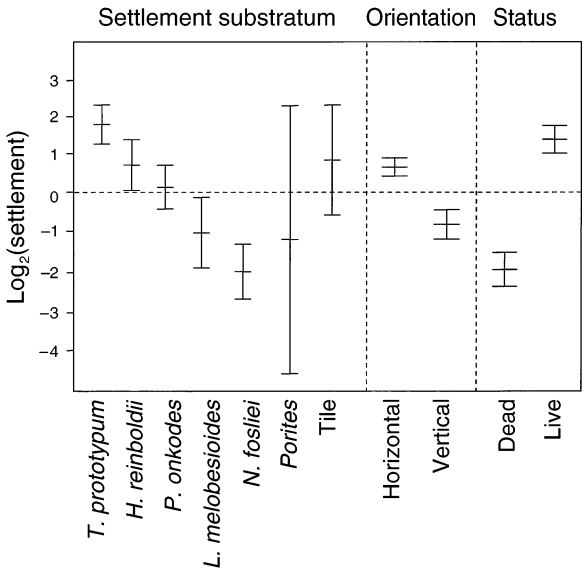

FIG. 1. Partial effects plots of settlement densities of coral larvae, showing the effects of various substrata (live and dead crustose coralline algae species (CCA) and inorganic substrata) at horizontal and vertical orientation (Experiment 1). The effects are plotted on $\log_2$ scale, and thus an increase or decrease by one unit corresponds to a doubling or halving in settlement numbers, respectively (e.g., settlement on live CCA is $2^{3.2} = 9.2$ times as high as on dead CCA). Error bars represent ± 1 SE.

2). Mortality was 100% on *N. fosliei* after three days, and <50% on two other substrata (*T. prototypum* and inert tile) after 22 days (Fig. 2). The log-linear analysis showed strong differences between substrata ($F_{7,584} = 23.04$, $P < 0.001$) and moderate differences due to days ($F_{(2,584)} = 5.50$, $P = 0.007$), orientation ($F_{(1,584)} = 5.48$, $P = 0.020$), and the interaction between substrata and days ($F_{(13,571)} = 4.47$, $P < 0.001$). This interaction was evident in the crossing of survival curves (Fig. 2), but

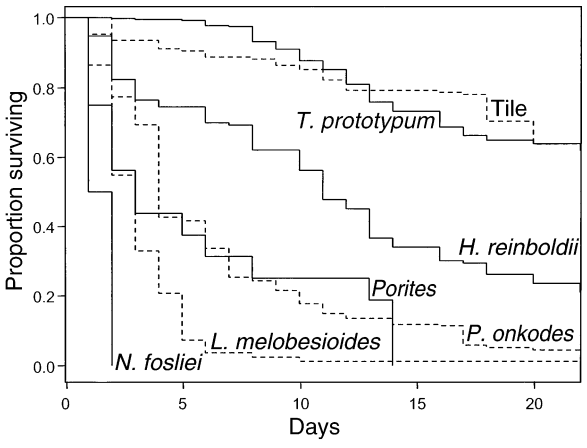

FIG. 2. Survival plots of coral settlers over 22 days (Experiment 2). The individual profiles correspond to proportions of surviving settlers on the five crustose coralline algae species and the two control substrata (*Porites* and tile) over time.

was not particularly strong; thus averaged effects for substratum and days were obtained by dropping the interaction from the model. The probability of mortality (hazard) varied greatly across the five species, and was 36.5 (20.1, 66.1) times higher for *N. fosliei* than for *T. prototypum*: the latter did not differ from tile (Fig. 3). For orientation, vertical tiles exhibited higher relative hazards (1.49, (1.13, 1.97)), and the relative hazard increased over the first 10 days but plateaued thereafter. After 240 days, survival of coral recruits on tiles and on *T. prototypum* was 20.1% (6.4, 42.0) and 24.2% (8.2, 52.1), whereas no survivors were observed on any of the other CCA species.

Three types of spat mortality were observed: (1) shedding of CCA thallus layers, thereby removal of the spat (see Plate 1), (2) overgrowth of the spat by CCA, and (3) mortality agents independent of visible/obvious CCA influences as estimated by mortality on the terracotta tiles (i.e., skeleton present, spat itself dead). Sloughing was seen in three CCA species: *N. fosliei*, *P. onkodes*, and *H. reinboldii*. In all colonies of *N. fosliei*, the sloughing of large sheets of epithallial cells removed >50% of the thallus in single events (see Plate 1; also see Appendix: panel A). In contrast, small flakes or individual cells of thallus filaments were continuously shed in *P. onkodes* and *H. reinboldii*, resulting in continuous loss of spat over the course of the first five days (Fig. 2). After 22 days, the overall post-settlement mortality was greater in *P. onkodes* than in *H. reinboldii*, as some proportions of the surfaces appeared to remain unshed over extended periods of time

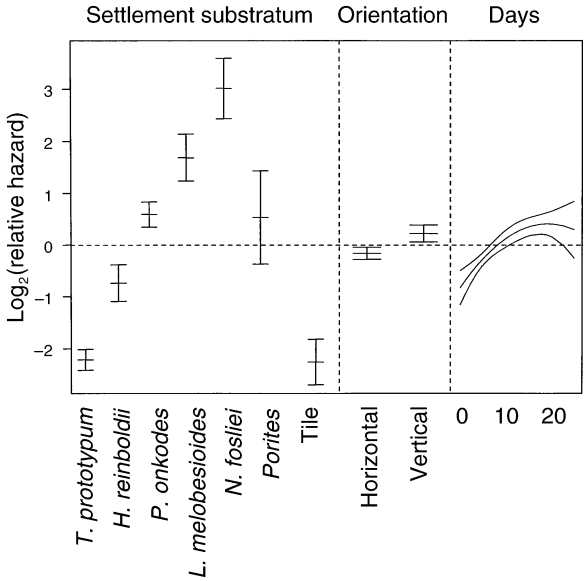

FIG. 3. Partial effects plots for relative hazard of coral spat (Experiment 2). The effects are for the substrata (crustose coralline algae species and inorganic substrata), orientation (horizontal and vertical), and the number of days since the start of the experiment. The effects are plotted on $\log_2$ scale; for interpretation see Fig. 1. Error bars represent ± 1 SE.

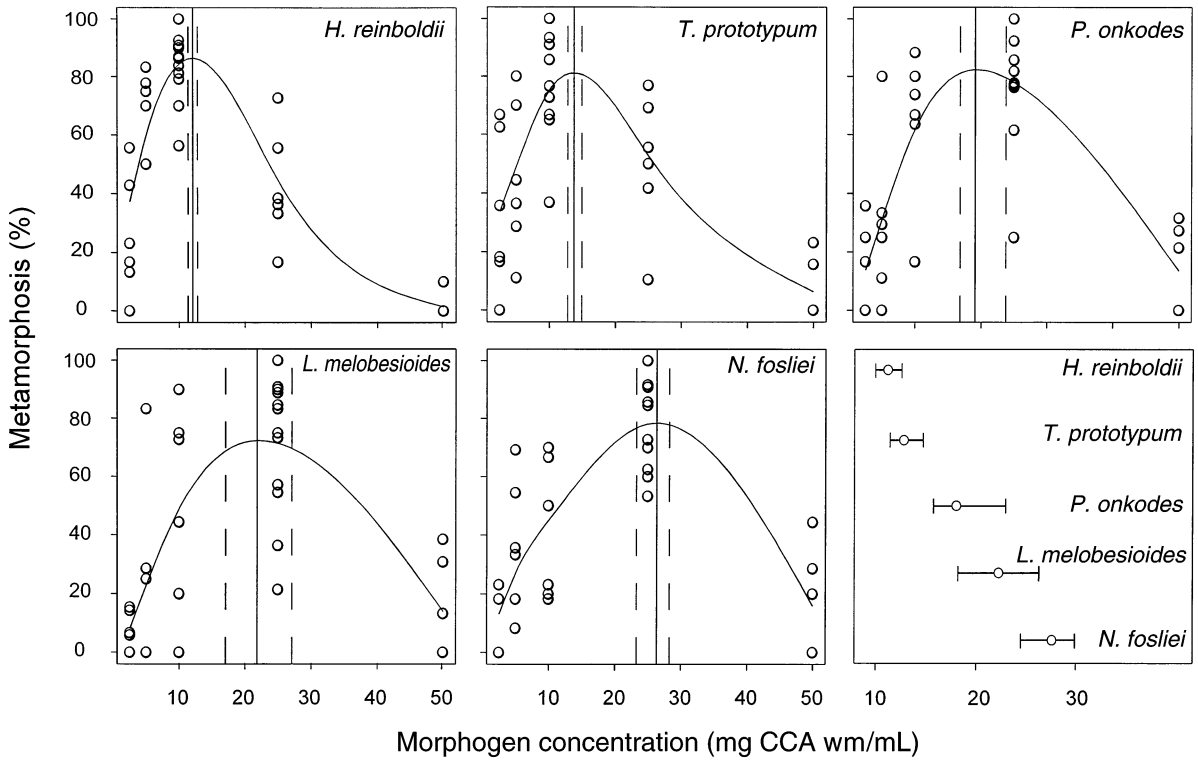

FIG. 4. Settlement and metamorphosis of coral larvae in response to concentrations of methanol-soluble extracts of crustose coralline algae (extract concentration based on mg CCA wet mass [wm]/mL seawater; Experiment 3). Smoothing splines were fitted to the response data, solid vertical lines indicate curve maxima (determined by bootstrapping analyses), and dashed lines are 10% and 90% confidence percentiles. Nonoverlapping bars in the final summary plot indicate significant differences ($P < 0.01$) between species in the locations of the maxima.

in the latter species. Although *L. melobesioides* was not observed shedding, the initial drop in density during the first three days suggests that the spat may have been lost in an unrecognized sloughing event. *T. prototypum* was also not observed to shed.

Overgrowth was another observed cause of mortality in coral spat. *T. prototypum* and *P. onkodes* were able to completely overgrow spat aged 7–30 days (0.5–2 mm in diameter), employing two distinctly different strategies. In *P. onkodes*, the growing margin grew up and over the spat (Appendix, panel C). In *T. prototypum*, the growing margins approached from all directions in very thin layers, surrounding the spat and reaching around/above the basal plate of the coral until they had completely overgrown the entire coral (Appendix: panel B).

Experiment 3: Larval metamorphosis in response to CCA morphogen concentrations.—Extracts of each of the five species of CCA induced settlement and metamorphosis in *A. millepora* larvae, confirming that methanol-soluble morphogens were present in all CCA species tested. The maximum level of metamorphosis occurred at different concentrations for the five species (Fig. 4, permutation test, $P < 0.001$), with levels of induction peaking at extract concentrations equivalent to 10–25 mg CCA/mL (Fig. 4). The maxima for *H. reinboldii* and *T. prototypum* induction occurred at lower extract concentrations (10 mg CCA/mL) than the remaining three species (25 mg CCA/mL), whereas there were no differences within each of these two groups. At low to medium concentrations, larvae were actively swimming and appeared healthy, whereas at the highest concentration used (50 mg CCA/mL), some of the larvae appeared unusually elongated and immobile. Control extracts (from terracotta, *Porites*, or no substratum) induced no metamorphosis over the concentration range examined.

Synthesis of the three experiments

Pairwise plots of the estimated effects for the five species showed strong positive relationships between CCA extract-induced metamorphosis, settlement, and survival (Fig. 5). High levels of coral settlement were related to high levels of survival (low relative hazards, $r = 0.99$, $P < 0.001$) and to high sensitivity to the corresponding CCA chemical inducer ($r = 0.91$, $P < 0.030$). Survival was highest on CCA species that induced highest levels of metamorphosis at low concentration of CCA extracts ($r = 0.92$, $P < 0.026$). The least chosen settlement substrata required higher concentrations of the species-specific CCA extract and exhibited low post-settlement survival, indicating that

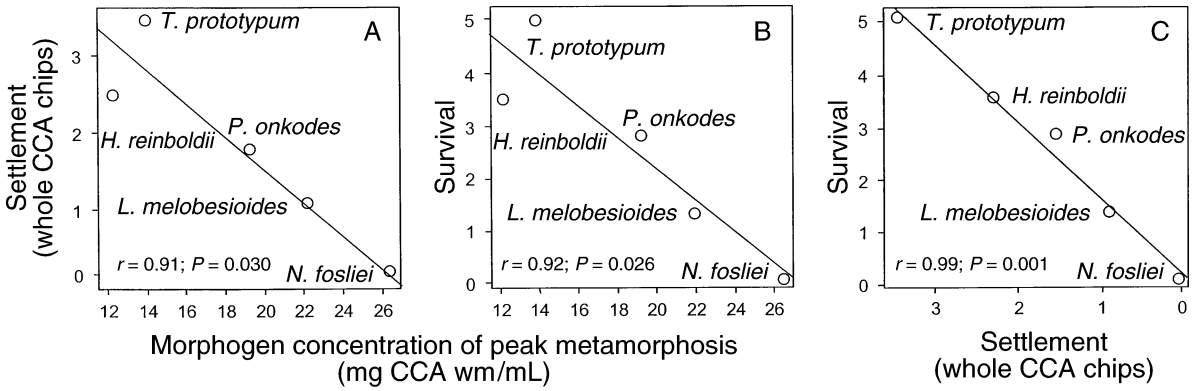

FIG. 5. Relationships between relative effects of sensitivity to chemical induction extract concentrations (Experiment 3), settlement (Experiment 1), and survival (Experiment 2) of coral larvae for the five species of crustose coralline algae (CCA). (A) Rate of settlement on whole live CCA fragments vs. sensitivity to CCA extract concentration. (B) Survival vs. sensitivity to CCA extract concentration. (C) Survival vs. settlement on whole, live CCA fragments. Sensitivity to chemical induction of metamorphosis was estimated as the concentration (%) at which peak induction occurred (Fig. 4), settlement effects were estimated as the relative probability of settlement ($\log_2$) (Fig. 1), and survival effects were estimated as the inverse of the relative hazard ($\log_2$).

early presettlement inducers and behaviors allow larvae to select and settle on substrata, thus enhancing their future survival probabilities.

DISCUSSION

CCA have previously been identified as preferred recruitment substrata for scleractinian corals (Morse et al. 1988, Raimondi and Morse 2000). Our research confirms this role for certain key CCA species of the Great Barrier Reef but not for other, more abundant, CCA species. We demonstrated that coral larvae are able to recognize and choose CCA species that have the least effective antifouling defenses. Active selection of permanent attachment onto certain substrata leads to an increase in survival over the first four weeks following settlement. The highest rates of settlement on *T. prototypum* coincided with lowest post-settlement mortality, and settlement was triggered by one of the lowest CCA extract concentrations. In contrast settlement induction and survival was lowest on the most abundant CCA species in the GBR, *N. fosliei* and *P. onkodes*. Therefore, a few select species of CCA can greatly contribute to controlling the fine-scale distribution patterns of corals within a reef ecosystem.

Settlement cues for coral larvae

Settlement rates in *A. tenuis* and *A. millepora* clearly differed depending on: (1) CCA species, (2) orientation of the substrata, and (3) whether the CCA was alive or not. Strong chemical inducers for settlement and metamorphosis (morphogens), such as the cell wall-bound polysaccharide identified from *H. borgesenii* (Morse and Morse 1991) should facilitate the selection of settlement sites that enhance post-settlement survival. Previous studies investigating the source and potency of coral morphogens have been performed using substrata or CCA extracts in isolation (Morse et al. 1988, 1996, Morse and Morse 1991, Heyward and Negri 1999). Those experiments demonstrate that extracts of most species of CCA tested contain morphogen(s) able to induce high levels of settlement and metamorphosis of coral larvae within 24 hours. Our results also show that, each of the CCA species tested contained a strong methanol-soluble morphogen, and that the potency of the CCA extract differed between species. The comparison of extract potencies may not reflect field behavior due to: potential differences between species in the solubility of active compounds, their surface area–mass ratios, and possible inhibition by co-extracted metabolites. When provided a choice of settlement substrata, larvae preferentially settled upon *T. prototypum*, the species that, along with *H. reinboldii*, exhibited the most potent CCA extract. Strong correlations between settlement preference on CCA fragments and extract potency were observed for all CCA species tested. These results suggest that coral larvae may be able to recognize subtle differences in chemical signatures or respond to different concentrations of morphogen on the surface of the CCA and use these signals when selecting their attachment site. Further research into the identity and specific concentrations of morphogen(s) associated with each of the CCA species is needed to confirm this.

Physical factors also contributed to determining the choice of attachment site. Settlement by *A. tenuis* larvae was significantly higher on horizontal opposed to vertical surfaces. This confirmed previous observations that larvae prefer upper surfaces for settlement, so long as these surfaces are free of sediment, algal growth, and grazing (Mundy and Babcock 1998). Physical conditions such as light and sedimentation are known to strongly influence settlement orientation in corals. For example, preferences shift from under surfaces in shallow water or at high light levels, to vertical surfaces

and then upper surfaces in deep water (Babcock and Mundy 1996, Baird and Hughes 2000). Again, this settlement behavior has selective advantages, despite the slower growth rate in a shaded position, as it reduces post-settlement mortality by grazing, algal growth, and sedimentation (Raimondi and Morse 2000).

The clear preference for larvae to settle on live CCA rather than dead CCA of the same species, indicates that chemical or biological properties of living CCA surfaces are more effective in inducing coral settlement, or that compounds released upon death inhibit settlement to some degree. This is supported by previous laboratory assays where larvae were induced to metamorphose by dead CCA but attached to the polystyrene walls of the test wells, instead of the dead CCA chips (Heyward and Negri 1999). Clean terracotta tiles do not contain chemical morphogens and need to be ''conditioned'' in seawater to enable the establishment of a microbial/algal biofilm before settlement takes place. The terracotta tiles used in these experiments were conditioned in unfiltered seawater for five days, and presented only a very immature biofilm; nevertheless they were a highly preferred settlement substratum, possibly due to early biofilm development on the tile surface (Webster et al. 2004). Alternatively, the larvae may have encountered water-borne CCA morphogens, or morphogens on nearby CCA (possibly through an amplifier pathway similar to that described in the larvae of the abalone *Halotis rufescens*; Morse and Morse 1984), and then moved along to metamorphose on the inert surface of the tile. Living CCA surfaces can aid in the survival of settlers, by excluding other space competitors, and, by providing protection from turf algae and the sediment entrapped in turf algae (Babcock and Mundy 1996, Ruiz-Zarate et al. 2000). However, while CCA can reduce the mortality of newly settled corals, these recruits also need to survive the defense strategies of the CCA, which include chemical deterrence, overgrowth, and sloughing.

Anti-settlement defense strategies of CCA

Allelopathic compounds are employed by certain CCA species as natural antifoulants (Masaki et al.1981, Suzuki et al. 1998, Degnan and Johnson 1999). In the present study, extracts from each CCA species induced maximum settlement levels of 80% at low to medium extract concentrations (Fig. 4); however, higher concentrations (50 mg CCA/mL) inhibited settlement and metamorphosis. Larval elongation and reduced searching activity at the high extract concentrations indicated the presence of either nonspecific settlement inhibitors co-extracted by methanol, or dissolved allelopathic compounds. As coral larvae successfully settled on the live fragments of each of the CCA species tested, it is unlikely that these species contain or release allelopaths against coral settlement in environmentally effective concentrations.

Overgrowth has been described in several CCA species as a strategy to outcompete fouling organisms including newly settled corals (Babcock and Mundy 1996, Dunstan and Johnston 1998; see *Results*), and adult corals (Antonius 2001). However, our data indicate that overgrowth by CCA plays a relatively minor role in the survival of coral settlers, as the number of overgrowth events was small. For example, after 6 mo the density of coral spat on *T. prototypum* was 24% of the initial level, despite observed overgrowth of recruits by this CCA, and not significantly different from that on inorganic terracotta tiles (20%).

Sloughing is a process by which some species of CCA shed off their upper epithallial layers thereby removing fouling organisms (Masaki et al. 1984, Keats et al. 1997). Sloughing of individual epithallial cells was the most effective antifouling strategy used by *N. fosliei*, *P. onkodes*, and *H. reinboldii*. Observations of incipient fouling in many species of living CCA, followed by the synchronous sloughing of whole epithallial layers (*Sporolithon* spp. and *Neogoniolithon* spp.) or a sloughing of individual epithallial cells in a spatially sequential fashion (*Neogoniolithon mammillare*) or diffuse fashion (*Porolithon* and *Hydrolithon*), indicate the importance of this process in CCA (Masaki et al. 1984, Johnson and Mann 1986). Similar antifouling strategies are widespread in marine and terrestrial plants; for example, seagrasses and laminarian kelps erode at the older apical regions to remove epibionts (Sand-Jensen and Borum 1991, Littler and Littler 1999). Scanning electron microscopy revealed that the epithalli of *T. prototypum* are usually intact, without peeling flakes. This anatomy and mode of growth appears to be a key to the high post-settlement survival on *T. prototypum*.

We are unable to compare the effectiveness of chemical defense against that of sloughing and overgrowth, as natural levels of allelopathic substances are unknown. The potentially allelopathic compounds co-extracted with the settlement inducers might primarily reduce the settlement of epibionts until the sloughing of epithallial cells of CCA surface occurs. Our combination of choice and no-choice settlement and survival experiments indicate that natural levels of allelopathy are insufficient to prevent coral settlement, that overgrowth is relatively ineffectual, and that sloughing is a highly effective strategy in preventing the survival of coral larvae on the five species of CCA tested.

Larval ranking of potential settlement substrata

We can now confirm that *T. prototypum* plays a critical role in influencing the fine-scale distribution patterns of coral recruits. Covering up to a few square centimeters, *T. prototypum* patches provide an excellent attachment surface for corals upon which competition and predation from other nonmotile reef organisms is low. However, *T. prototypum* comprises $<5\%$ of the CCA flora on GBR reefs (R. Steneck, E.

Turak, L. Harrington, and T. Done *unpublished manuscript*). So, rather than specializing in settlement exclusively onto a single uncommon CCA species, the larvae are able to actively recognize and select the next preferred substrata as suitable settlement sites, possibly keying into surface morphogens found in varying abundance or structure among CCA species (Fig. 5A). *Hydrolithon borgesenii* has been described as the key substratum for coral settlement in the Caribbean (Raimondi and Morse 2000). A similar species, *H. reinboldii*, is dominant throughout the tropical Indo-Pacific (Adey et al. 1982), and this species also contained a highly potent methanol-soluble morphogen and exhibited the second highest settlement and survival rates out of the remaining CCA species.

Habitat selection by planktonic larvae is critical to subsequent survival of sessile invertebrates, since location largely determines the environmental conditions experienced by later life stages (Keough and Downes 1982, Baird et al. 2003). Apart from recently disturbed and damaged reef surfaces, CCA offers a potentially favorable attachment site for disparate marine invertebrate taxa including coelenterates, which is stable and can facilitate survival and development to reproductive maturity. Among these settlers, the specificity of the interaction covers a spectrum, from species that manifest specificity for a particular species of CCA (Morse et al. 1996), to those requiring contact with any of a variety of CCA (Morse et al. 1988). We further demonstrated that coral larvae are able to actively recognize and rank different species of CCA as suitable settlement sites primarily based upon chemical signature and clearly reflecting the effectiveness of their antifouling strategies. This ranking process strongly supports the notion that settlement behavior (habitat selection) is adaptive and may be largely responsible for the fine-scale recruitment patterns observed in the field leading to a wider influence on coral reef structure.

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APPENDIX

A photograph showing examples of anti-settlement defenses in crustose coralline algae is available in ESA's Electronic Data Archive: *Ecological Archives* E085-120-A1.

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Male courtship signals and female signal assessment in *Photinus greeni* fireflies

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The evolutionary dynamic of courtship signaling systems is driven by the interaction between male trait distributions and female preferences. This interaction is complex because females may choose mates based on multiple components of male signals, and female preference functions may vary depending on mate availability, female reproductive state, and environmental conditions. In *Photinus* fireflies (Coleoptera: Lampyridae), flying males emit bioluminescent flash signals to locate sedentary females, which reply selectively to attractive male flash signals with their own response flash. In this study, we first examined temporal variation in the paired-pulse flash patterns produced by *Photinus greeni* males in the field and found significant among-male variation (~70% of total variation) in interpulse intervals (IPIs). There was no significant relationship between male IPI and spermatophore size, suggesting that *P. greeni* male courtship signals do not provide females with reliable indicators of male material resources. In laboratory playback experiments, we presented *P. greeni* females with simulated flash signals to assess how IPI and pulse duration independently affected the likelihood of female flash response. We also examined the effects of female body mass and time during the mating season on female preference functions, hypothesizing that females would be less discriminating when they were heavier (more fecund) and when mate availability declined. We found that *P. greeni* females discriminated among signals within their species' range based primarily on flash pattern IPI. Neither the time during the mating season nor female weight altered female preference functions for IPI, although season did influence female response to pulse duration. These results reveal that *P. greeni* females discriminate among conspecific males based primarily on male IPIs, the same signal character previously shown to be important for firefly species recognition. Field playback experiments indicated that female responsiveness peaked near the average IPI given by males at different ambient temperatures, suggesting that fireflies exhibit temperature coupling similar to that seen in many acoustically signaling animals. *Key words:* bioluminescence, mate choice, multiple cues, preference functions, sexual selection, temperature coupling. *[Behav Ecol 17:329–335 (2006)]*

The direction and intensity of sexual selection on male courtship signals depend on the interaction between male trait distributions and female preference functions (Andersson, 1994; Wagner, 1998). Several hypotheses have recently been proposed for how females might combine information from multiple male signals to recognize or assess potential mates (reviewed in Candolin, 2003; Hebets and Papaj, 2005). For example, multiple signals (sensu Maynard Smith and Harper, 2003) might provide females with redundant information about male quality (backup messages hypothesis) or with information about distinct aspects of male genetic quality or phenotypic condition (multiple messages hypothesis). Additionally, different signals may be used for purposes of species recognition and mate quality assessment. To begin distinguishing among hypotheses concerning multiple signals, we need to examine how females perceive and respond to courtship signal components and determine what information such signals convey.

Theoretical and empirical studies indicate that female preferences are not fixed but rather are phenotypically plastic behaviors that may depend on a variety of factors, including female nutritional status, mating history, mate availability, and temperature (Fawcett and Johnstone, 2003; Gibson and Langen, 1996; Jennions and Petrie, 1997; Lynch et al., 2005; Pitcher et al., 2003; Real, 1990). The phenomenon of temperature coupling represents an example of linked variation between male signals and female preference. In many acoustically signaling ectotherms, temperature dependence of male courtship signals is matched by a concordant change in female preferences (e.g., Gerhardt, 1978; Pires and Hoy, 1992; but see Ritchie et al., 2001). Such temperature coupling allows females to recognize conspecific male signals independently of variation in environmental temperatures. However, few studies have examined whether temperature coupling occurs in the bioluminescent signaling systems of fireflies (but see Carlson et al., 1976).

Because courtship in nocturnal *Photinus* fireflies is based on highly visible bioluminescent signals, these insects are particularly amenable to studies of female choice. Previous work has examined variation in male flash signals and female preference in several *Photinus* firefly species (Branham and Greenfield, 1996; Cratsley and Lewis, 2003; reviewed in Greenfield, 2002; Lewis et al., 2004). In *Photinus ignitus*, males emit a single-pulse courtship signal, and females respond preferentially to longer duration signals. Early in the mating season, *P. ignitus* males with longer signal durations also provide larger nuptial gifts (spermatophores) to females during mating. Therefore, male courtship signals in some *Photinus* may allow females to assess the material resources provided by a potential mate. Previous studies have also demonstrated that female responsiveness in *Photinus* fireflies is context dependent, influenced by both a female's mating history and her nutritional status (Cratsley and Lewis, 2003). *Photinus* fireflies thus offer a useful model for understanding how multiple male signal characters influence female choice as well as for exploring plasticity of female preference functions.

This study was designed to characterize variation in the bioluminescent courtship signals of field-recorded *Photinus greeni* males and to determine whether male flash traits are correlated with direct benefits provided by male spermatophores.

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We also used both field and laboratory photic playback experiments to assess female preference for two male signal traits, interpulse interval (IPI) and pulse duration, when these signal traits were varied independently. Finally, we examined the plasticity of female preference functions in response to female body mass, mate availability, and environmental temperatures.

METHODS

Study organism and site

In most *Photinus* fireflies, roving males produce sexual advertisement flashes to locate sedentary females, which respond to attractive male flash signals with their own bioluminescent flashes (Carlson and Copeland, 1985; Lloyd, 1966). Courtship periods typically last 1–2 h each night, during which individuals of both sexes have the opportunity to sample many potential mates, although both sexes mate only once each night (Lewis and Wang, 1991; Wing, 1985). We chose to investigate male courtship signals and female response of *P. greeni* fireflies. Compared to other *Photinus* species (Branham and Greenfield, 1996; Cratsley and Lewis, 2003), relatively little is known about male signal variation or female preferences for *Photinus* species like *P. greeni*, in which males emit courtship signals consisting of paired pulses (but see Buck J and Buck E, 1972; Buck and Case, 1986; Carlson et al., 1976, 1977). In addition, *P. greeni* is part of the *consanguineus* species complex consisting of several morphologically indistinguishable species differentiated only by their courtship flash behavior (Lloyd, 1969). Three species have been described based on the time interval between the paired pulses comprising their male courtship signals: *Photinus consanguineus* has a short, ~0.5-s IPI, followed by *P. greeni* with ~1.0–1.5 s, and *Photinus macdermotti* with ~2.0-s IPI. The *consanguineus* species complex is broadly distributed across the eastern US, with *P. greeni*, *P. macdermotti*, and *P. consanguineus* showing extensive geographic overlap (Lloyd, 1966, 1969). Female assessment of male flash signals within this species complex may function not only in species recognition (Lloyd, 1966, 1969) but also in mate quality discrimination.

In this study, experiments were conducted and fireflies were collected at the Smith-Andover field in Lincoln, Massachusetts, USA (42° 26′ N, 71° 18′ W) during June–July 2004. *P. greeni*, the only species in the *consanguineus* complex present at our site, was first observed flashing on 8 June (defined as day 1 of the mating season) and was last found on 24 July (day 43). *P. greeni* is a dusk-active species (Buck J and Buck E, 1972; Lloyd, 1969); males begin signaling ~15 min after sunset, and the male flight period lasts ~45 min. By 90 min after sunset, the majority of *P. greeni* dialogs have ceased. As in other *Photinus* species (Cratsley and Lewis, 2005; Lewis and Wang, 1991), *P. greeni* male availability declines over the course of the mating season.

Male flash signal variation

To characterize variation in courtship signals, flash phrases produced by individual *P. greeni* males were video recorded at 30 frames per second (fps) using a Sony TRV80 digital camcorder during days 1–20 of the mating season. Males were videotaped until 15 male phrases were recorded, the male stopped flashing, or the male was lost. After 15 phrases were recorded, males were collected to prevent rerecording. During videotaping, we noted the time and ambient air temperature at the male's flight height.

From the video recordings, we measured two male signal characters, pulse duration and IPI (the time interval between the paired pulses that make up each flash phrase of *P. greeni* males); we did not measure male flash pattern repetition rate because this character is highly dependent on signaling context. IPIs for each male were measured with frame-by-frame analysis of digitized video using iMovie 4. This IPI was determined as the number of frames between the first visible light of the first male pulse and the first visible light of the second male pulse, multiplied by the time span of each frame (33 ms). This method of measuring male *P. greeni* IPI yielded a maximum error of 6.6%. Only males that emitted at least four video recorded flash phrases were included in our analysis, which included 221 male flash phrases from 30 *P. greeni* males.

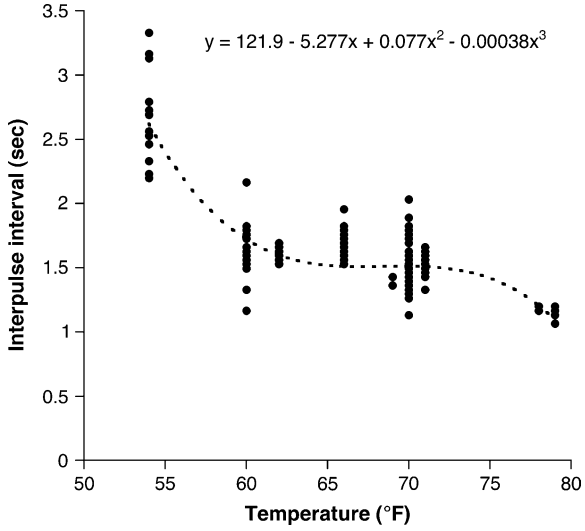

Figure 1
Dependence of field-recorded male *Photinus greeni* courtship IPI on ambient air temperature ($r^2 = .724$). Male IPIs were video recorded in the field ($n = 221$ flash patterns), and a polynomial regression was used to temperature adjust all male IPIs to 70°F (see Figure 2).

For measurements of pulse durations, we increased temporal resolution by deinterlacing video recordings to yield a frame rate of 60 fps. This allowed us to measure male pulse durations in 16.67-ms increments. Because this results in a possible overestimate of up to 50% for the shortest pulse durations (~70 ms), we report only the range of *P. greeni* pulse durations measured at 70°F, which represents the testing range used in our laboratory studies of female preference.

Temperature affects the mating signals of many ectotherms, including fireflies (Carlson et al., 1976; Lloyd, 1966). To assess the variation in IPI among males recorded in the field at different ambient temperatures, we temperature adjusted each IPI to a common temperature of 70°F, the ambient temperature used during laboratory testing of female preferences. Field-recorded IPIs were temperature adjusted by fitting a polynomial regression (Figure 1, $r^2 = .724$) and adding the residual from this regression to the mean IPI at 70°F. We examined variation within and among *P. greeni* males in their temperature-adjusted IPI using one-way ANOVA. We also examined the possible relationship between male signal characteristics by comparing each male's average temperature-adjusted IPI to his average temperature-adjusted pulse duration. To avoid including pulse duration in both variables, we first subtracted pulse duration from the IPI measurements and then examined the correlation between signal traits.

We also assessed variation in flash signals produced by *P. greeni* males during three distinct stages in their nightly activity period: warm up, patrolling, and dialoguing. Warm-up flashes were defined as those flashes produced by males perched at the top of grass blades at the onset of the male

flight period (approximately up to 30 min after sunset). Patrolling flashes were defined as advertisement signals given by flying males. Dialoguing flashes were defined flashes as given by males that were engaged in courtship dialogs with females. We compared male IPI (temperature adjusted as described above) across the three stages using a Kruskal-Wallis nonparametric test.

We examined whether courtship signals in *P. greeni* are correlated with the mass of males' spermatophores, which provide a direct fecundity benefit to females (Rooney and Lewis, 2002). Due to the difficulty of measuring both male signals and spermatophore size, for this analysis we combined data from 2004 and 2005 mating seasons. In both years, males were collected during the first 10 days of the mating season; we focused on early mating season because spermatophore size is known to decline across successive matings (Cratsley et al., 2003). After each male's flash pattern was video recorded (2–15 flash phrases for each male), males were allowed to mate with assigned females, and spermatophores were dissected from female reproductive tracts shortly after transfer (van der Reijden et al., 1997). Spermotophores were rinsed briefly in distilled water, dried in a desiccator for 24 h, and then weighed to the nearest 1 μg on a Mettler MT5 microbalance. In *P. ignitus,* spermatophore mass has been shown to be influenced by male body size (Cratsley and Lewis, 2003; Cratsley et al., 2003), so we accounted for possible covariation by including male mass in a multiple linear regression analysis to see if male flash IPI predicted male spermatophore mass.

Female preference functions: laboratory experiments

Flash characteristics affecting female response were identified through experiments in which *P. greeni* females were presented sequentially with courtship signals differing in either IPI or pulse duration. Field-collected females were weighed to the nearest 0.1 mg and kept on a reversed 8:16 h light:dark cycle. To examine seasonal changes in female responses, we compared *P. greeni* early-season females (collected on days 7–11 of the mating season under male-biased sex ratios) to late-season females (collected on days 21–39 under female-biased sex ratios). Females' previous mating histories were unknown, although late-season females had likely mated at least once.

Females were tested individually shortly after artificial dusk (lights dimmed to ~20 lux), at temperatures maintained between 70°F and 72°F. Flash pulse duration and IPI were varied separately, with five levels of each presented to females in a latin square design to control for presentation order effects. Females were positioned 24 cm from the output LED (570 nm, Ledtronics Inc., Torrance, CA) of a programmable flash generator that produced square pulses near the peak wavelength of male *P. greeni* flashes (572 nm; Case, 1984). To examine the effects of different pulse durations on the likelihood of female response, each female ($n = 56$) was presented with 20 flash phrases, consisting of four replicates each of five different pulse durations: 50, 80, 100, 120, and 150 ms, keeping a constant 1.0-s IPI, with 10 s between flash phrases. Using a similar design to examine the effects of different IPIs on female response, we presented females ($n = 49$) with 20 flash phrases, consisting of four replicates each of five different IPIs: 0.8, 1.0, 1.2, 1.4, and 1.6 s, with a constant 100-ms duration and 10 s between flashes. Most females ($n = 45$) were given both trials on the same night, in random order (either duration or IPI first) with 40 min between trials. As flash responses of *P. greeni* females are all or nothing, females were scored as responding if they produced a response flash at the characteristic 0.8-s delay following the simulated male phrase. Females that failed to respond to at least one stimulus phrase were omitted from the analysis.

To analyze female preference functions (Wagner, 1998), we modeled female response as a correlated binary response variable (repeated measures on each female) with generalized estimating equations (GEE) using PROC GLM (SAS version 9.1) and an exchangeable correlation structure (Quinn and Keough, 2002). Because firefly courtship matches assumptions of mate choice models involving sequential search with time constraints (Fawcett and Johnstone, 2003; Johnstone, 1997; Real, 1990), we tested the prediction that females will show lower mate acceptance thresholds later in the mating season as mate availability declines. We also tested the prediction that females with lower residual reproductive value (fewer eggs) would have lower acceptance thresholds by examining how preference functions changed with female body mass (*Photinus* female mass is correlated with egg count; Cratsley and Lewis, 2005). Separate GEE models were used for duration and IPI trials to examine how each signal characteristic, presentation order, female body mass, and time during mating season (early versus late season) affected the probability of female flash response.

Female response to simulated courtship flashes: field experiments

To investigate whether laboratory measurement of female preference functions reflected female behavior under field conditions, we presented *P. greeni* females with simulated flash phrases differing only in IPI. These trials were conducted during the latter part of the mating season (days 22–38) on females in situ that were located immediately prior to testing. The output LED of the flash generator was positioned in view of and ~30 cm away from perched females ($n = 34$), and the presence or absence of a female response was noted for 20 simulated courtship flash phrases (four replicate flash phrases for each of five different IPI levels, with constant 100-ms pulse duration and 10 s between consecutive phrases). The levels presented were adjusted to ambient air temperatures recorded near each female and bracketed the mean male IPI observed at that temperature (Figure 1): $\overline{X}$ minus 0.6, 0.4, and 0.2 s, $\overline{X}$ and $\overline{X}$ plus 0.2 s. During testing, any males that came within 2 m of the female were removed, and after testing females were collected for body mass measurements as described above.

Response of these *P. greeni* females tested in situ was again modeled as a correlated binary response variable using GEE to examine the effect of IPI, presentation order, female body mass, and ambient temperature on the probability of female flash response. Only females that were tested between 60°F and 71°F were included in our analysis as the pulse duration of *P. greeni* male signals remains fairly constant across this temperature range (unpublished data). Again, any female that failed to respond to at least one stimulus phrase was omitted from the analysis.

RESULTS

Male flash signal variation

When flash patterns of *P. greeni* males were measured in the field, IPIs averaged 1.51 ± 0.18 s (mean ± 1 SD, temperature adjusted to 70°F; Figure 2). IPIs differed significantly among males (ANOVA: $F_{29,191} = 17.88$, $p < .0001$), with among-male variation representing 69.8% of the total variation. When we compared flash signals given by males during different courtship stages, there was no significant difference in the IPI of signals used by males during warm-up, patrolling, and dialoguing stages (Figure 3; Kruskal-Wallis $\chi^2 = 1.81$, 2 df, $p = .40$). For *P. greeni* courtship signals measured at 70°F, pulse durations

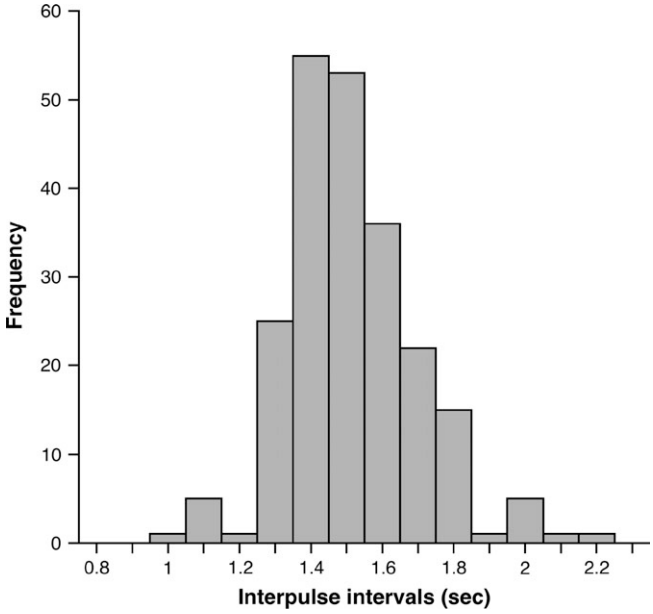

Figure 2
Frequency distribution of male IPIs (temperature adjusted as described in methods) from field-recorded *Photinus greeni* males ($n = 221$ two-pulsed flash phrases recorded from 30 males).

ranged from 67 to 233 ms ($n = 81$ pulses). For individual males ($n = 23$), there was no correlation between average pulse duration (temperature adjusted) and average IPI (minus duration, temperature adjusted; $r^2 = .319$, $p = .14$).

P. greeni male spermatophore mass ranged from 55 to 191 μg and was not significantly related either to male IPI (multiple regression, $n = 15$ males, $t = 0.19$, $p = .853$) or to male mass ($t = 1.10$, $p = .295$).

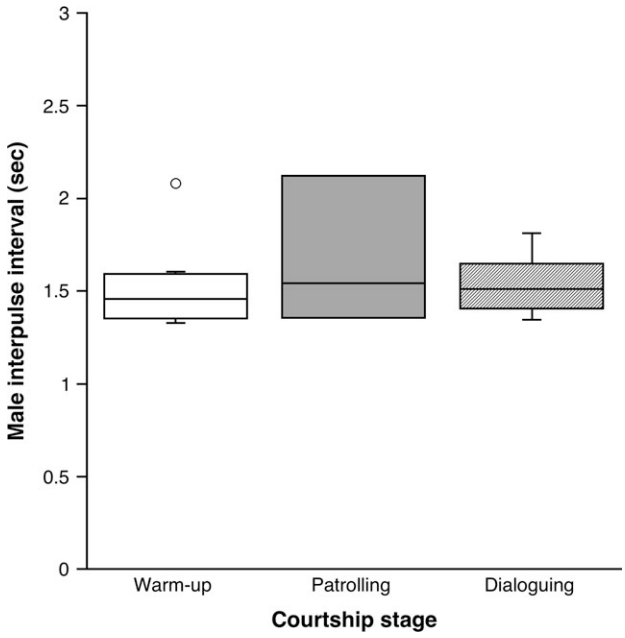

Figure 3
Box plots comparing mean IPIs from field-recorded *Photinus greeni* males during three courtship stages (warm up, $n = 12$ males; patrolling, $n = 4$ males; dialoguing, $n = 14$ males). In each box plot, the mid line shows the median, the box represents the middle 50% of data values, whiskers extend to 10th and 90th percentiles, and the circle indicates an outlying data point.

Female preference functions: laboratory experiments

Females showed highly significant differences in how responsive they were to simulated courtship signals with differing IPIs ranging from 0.8 to 1.6 s presented in a latin square design in the laboratory (Figure 4a, Table 1). Females showed maximum flash responses to signals with IPIs of 1.0 s, with female responsiveness declining below 10% for signals with 1.4- and 1.6-s IPIs. The likelihood of female response was not affected by presentation order, by whether females were collected early versus late in the mating season, or by female body weight (Table 1).

Females also showed highly significant differences in responsiveness to different pulse durations, although responsiveness remained between 32% and 70% for signals with pulse durations ranging from 50 to 150 ms (Figure 4b, Table 1). In these duration trials, early-season females were significantly more responsive than late-season females, and female responsiveness was also influenced by presentation order. Female body mass did not influence female response to pulse duration.

Female preference functions: field experiments

Although there was no effect of temperature on female responsiveness, there was a significant interaction between temperature and IPI (Figure 5, Table 2). Females tested at low ambient temperatures (60°F–65°F) were much more responsive to longer IPIs compared to females tested at high ambient temperatures (66°F–71°F). Neither female body weight nor presentation order (increasing or decreasing IPI) affected female response.

Differences in female response profiles to signal IPI between laboratory experiments (Figure 4) and field experiments (Figure 5) could reflect differences in female body mass or time during the mating season. Laboratory-tested females were collected earlier during the mating season and weighed significantly more (27.4 ± 1.0 mg) than field-tested females (20.8 ± 1.0 mg) (Kruskal-Wallis $\chi^2 = 16.96$, 1 df, $p < .0001$).

DISCUSSION

This represents the first comprehensive study of intraspecific variation in male bioluminescent courtship signals, female preference functions, and possible benefits of female choice for fireflies in the *P. consanguineus* complex. We found considerable among-male variation in both temporal characteristics of *P. greeni* flash patterns, IPI and pulse duration. Although males in the sibling species *P. macdermotti* emit different flash patterns while patrolling and courting (Carlson et al., 1976), we found that signal IPIs were consistent across three courtship stages for *P. greeni* males. Our results also demonstrate that female *P. greeni* fireflies discriminate among conspecific males based mainly on their IPIs. When we independently varied signal characteristics of male courtship flashes in laboratory photic playback experiments, the slope of the female preference function to different IPIs (Figure 4a) was steeper than the slope of the female preference function to different pulse durations (Figure 4b). However, although *P. greeni* females were less sensitive to pulse duration, this characteristic may still play a role in female signal assessment.

In *Photinus consimilis* fireflies, where male signals consist of multiple pulses, females were also less sensitive to male pulse duration compared to pulse rate (inverse of IPI, Branham and Greenfield, 1996; Greenfield, 2002). However, females responded to differences in pulse duration in *P. ignitus*, where

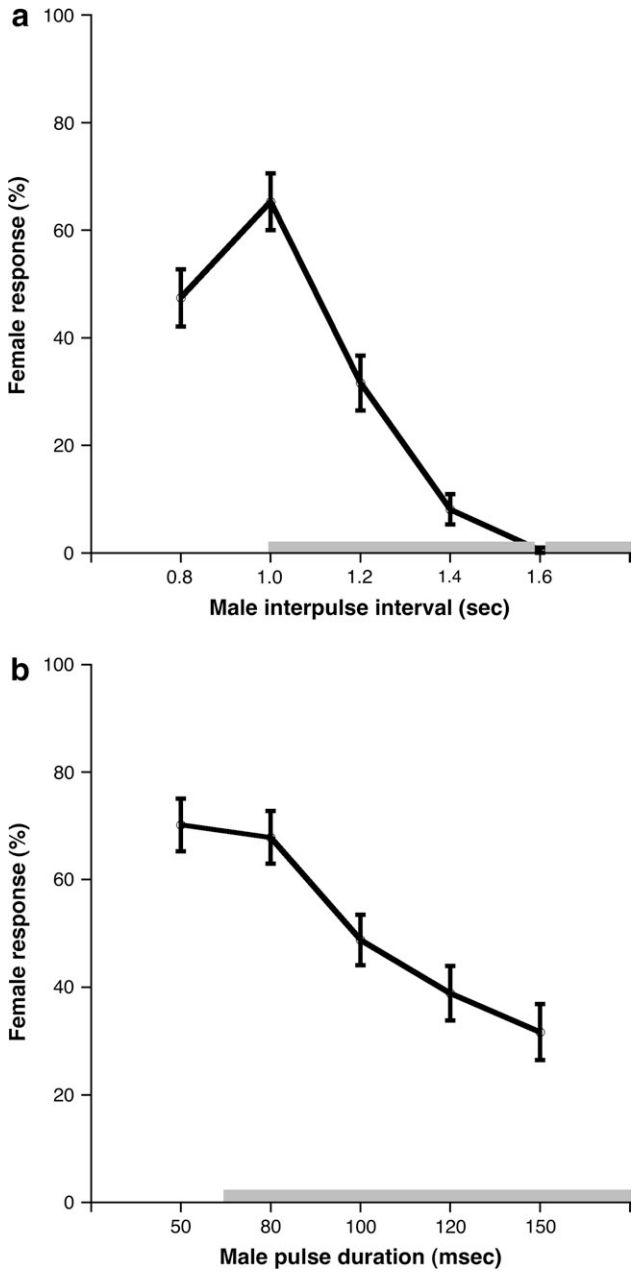

Figure 4
Preference functions of *Photinus greeni* females to simulated male signals presented in a latin square design in laboratory tests conducted at 70°F–72°F. Each female was presented with 20 flash phrases (four repetitions of each of five levels, with 10 s between consecutive flash phrases). Data shown as mean (±1 SE) percent of flashes to which females responded during (a) IPI trials ($n = 49$ females tested with five different IPIs and constant 100-ms pulse duration) and (b) pulse duration trials ($n = 56$ females tested with five different pulse durations and constant 1.0-s IPI). Shaded blocks indicate observed range of male IPIs and pulse durations for this *P. greeni* population.

Table 1
Statistical results from separate GEE analyses examining how the flash responsiveness of *Photinus greeni* females is influenced by courtship signal characteristics of IPI and pulse duration, female body weight, season (early versus late mating season), and presentation order

Source	df	Chi square	*p*
IPI trials			
IPI	4	95.53	<.0001
Female weight	1	2.06	.1517
Season	1	2.01	.1559
Presentation order	4	10.86	.1303
Pulse duration trials			
Pulse duration	4	37.46	<.0001
Female weight	1	0.11	.7448
Season	1	8.29	.0040
Presentation order	4	18.45	.0010

In both experiments, *P. greeni* females ($n = 49$ for IPI trials, $n = 56$ for pulse duration trials) were each tested with 20 flash phrases (four each of five signal parameter levels) in the laboratory at 70°F–72°F.

males emit a single-pulse courtship signal (Cratsley and Lewis, 2003). Only a few previous studies have examined female preferences within those *Photinus* species where males emit two-pulsed courtship signals. Our finding that *P. greeni* females discriminate among male signals mainly on the basis of IPI is consistent with results from earlier studies of *P. macdermotti* (Carlson et al., 1976, 1977) and *P. greeni* (Buck J and Buck E, 1972; Buck and Case, 1986; Lloyd, 1969), although these studies did not control for the effects of presentation order. Thus, although *Photinus* species differ in which temporal aspects of male courtship signals are important, each species' females appear to rely primarily on a single aspect of male flash signals, rather than multiple flash characteristics, to assess males as potential mates. Because this study focused only on flash signals, we cannot rule out the possibility that females might also assess male traits using other sensory modalities. For example, in addition to assessing flash IPIs, after contacting a male females might use chemical signals, such as cuticular hydrocarbons, to gain further information about mate quality.

Sexual signals can function in both species recognition and mate choice (reviewed in Andersson, 1994), and these might involve either the same or different signals. For example, in the acoustic signals of *Hyla versicolor* and *Hyla chrysoscelis* tree frogs, females use the pulse rate of male calls for species recognition, while call duration is used for mate assessment (Gerhardt, 2001). In contrast, *P. greeni* females appear to discriminate among conspecific males based mainly on their IPI, a signal character that Lloyd (1966, 1969) has shown to be also important in species recognition within the *consanguineus* complex. When *P. consanguineus* and *P. macdermotti* females were tested in the field, they failed to respond to flash signals with IPIs outside the range of their own species (Lloyd, 1966). Thus, within the *consanguineus* species complex, female preference functions for male IPI are likely to be shaped simultaneously by the dual processes of species recognition and mate quality assessment.

Many models of sexual selection are based on male mating signals that act as reliable indicators of male phenotypic or genetic quality (Andersson, 1994). During mating, *Photinus* males transfer a protein-rich spermatophore to females (van der Reijden et al., 1997), and these spermatophores have been shown to increase female fecundity (Rooney and Lewis, 2002). In *P. greeni*, we found no relationship between male IPI and spermatophore size, which suggests that in this species, male IPIs are uninformative with respect to male material resources. However, we cannot eliminate the possibility that other flash characteristics (pulse duration) may indicate male resources, as has been shown for the congener *P. ignitus* (Cratsley and Lewis, 2003). It is also possible that *P. greeni* courtship signals reflect other aspects of male phenotypic or

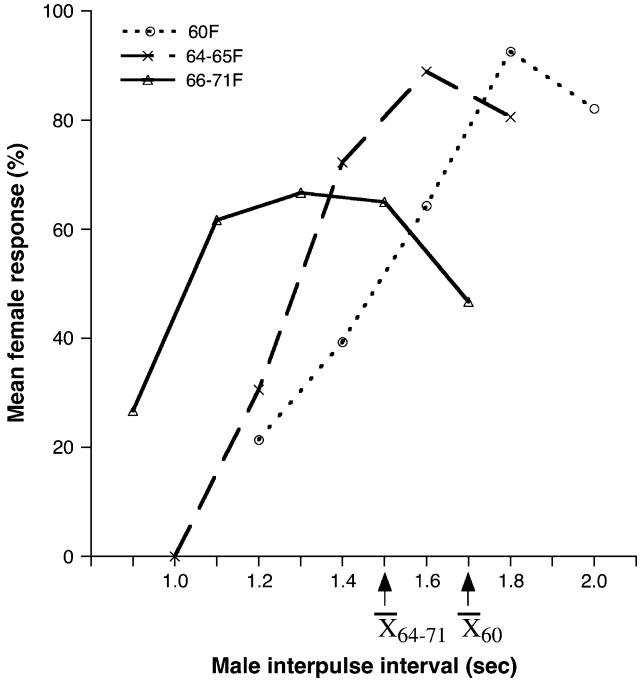

Figure 5
Preference functions of *Photinus greeni* females tested in the field at different ambient temperatures: 60°F ($n = 7$ females), 64°F–65°F ($n = 9$), and 66°F–71°F ($n = 15$). Each female was presented with 20 flash phrases (four flash phrases at each of five different IPIs with constant 100-ms pulse duration). Mean female response is shown for females within each temperature range (error bars omitted for clarity of presentation). Arrows indicate observed population means for male IPIs for the temperature ranges at which females were tested.

genetic quality or that females assess additional traits during or after mating to correct mate choice errors (as proposed by Lewis et al., 2004).

Female preference functions are predicted to vary as costs of mate choice change with female condition or mate availability (Fawcett and Johnstone, 2003; Jennions and Petrie, 1997; Real, 1990). Previous studies of other *Photinus* species have measured female response to conspecific males in the field and have shown that the overall level of female response increases as the availability of potential mates decreases later in the mating season (Cratsley and Lewis, 2005; Lewis and Wang, 1991). In this study, we found no seasonal change in *P. greeni* female preference functions for different IPIs, although females collected early in the mating season were more responsive when tested with different pulse durations. In laboratory experiments, *P. ignitus* females that had mated or had been fed an artificial diet showed reduced response levels to simulated male signals (Cratsley and Lewis, 2003). However, our results indicated that female preference functions were unaffected by differences in female body weight. Thus, while changes in mate availability and female mass may affect the overall level of female responsiveness, this study suggests that the shape of female preference functions for male IPIs remains relatively constant even as costs of mate choice change.

Table 2
Statistical results from GEE analysis for the effects of courtship signal IPI, female condition index, ambient temperature, and presentation order on the flash responsiveness of *Photinus greeni* females measured in the field at temperatures ranging from 60°F to 71°F ($n = 34$ females each tested with 20 flash phrases)

Factor	df	Chi square	p
IPI	4	17.00	.002
Female weight	1	1.03	.310
Ambient temperature	1	1.54	.215
Presentation order (increasing or decreasing IPI)	1	0.12	.730
Temperature × IPI	4	15.13	.004

The acoustic signals of many ectotherms show strong temperature dependence in their temporal components (Gerhardt, 1978; Greenfield, 2002; Pires and Hoy, 1992). In contrast, we found that IPIs of *P. greeni* males were roughly constant across a relatively wide range of ambient temperatures. Our results differ from the linear temperature dependence found for the temporal features of other *Photinus* signals noted by Carlson et al. (1976) and Lloyd (1966), perhaps reflecting geographical differences between study populations. However, our results do indicate that the preference functions of *P. greeni* females for male flash signals change with ambient temperatures as we found a significant temperature × IPI interaction for females tested in the field across temperatures ranging from 60°F to 71°F (Figure 5, Table 2). Females tested in the field at different ambient temperatures showed highest responses near the average IPI displayed by males at that temperature. As suggested by Carlson et al. (1976), these results indicate that *P. greeni* fireflies exhibit some temperature coupling between male signals and female preference, as has previously been shown for acoustically signaling insects (e.g., Pires and Hoy, 1992).

Different preference functions for IPIs were found when females were tested in the laboratory (Figure 4) versus in the field (Figure 5). Females tested in the laboratory (70°F–72°F) showed their highest response to shorter IPIs compared to females tested in the field at similar temperatures (66°F–71°F). Possible explanations for this discrepancy include that field-tested females were tested later in the season and thus were more likely to have mated multiple times. Field-tested females also had significantly lower body mass, reflecting lower egg loads. It is also possible that female preference functions may involve an interaction between IPI and pulse duration. For instance, given the demonstrated female preference for shorter pulse durations and the increase in male pulse durations at lower temperatures, it is possible that the 100-ms pulse duration used in our field testing of females may have been more attractive to females at colder temperatures. Overall, the observed plasticity in female mating preferences may contribute toward the maintenance of variation in male signals.

In conclusion, these studies have demonstrated variation in two temporal characteristics of courtship flashes among male *P. greeni* fireflies. *P. greeni* females appear to discriminate among conspecific males based primarily on male IPIs, the same signal character previously shown to be important for species recognition. There was no significant relationship between male IPI and spermatophore size, suggesting that *P. greeni* male courtship signals do not provide females with reliable indicators of male material resources. Female preference functions changed significantly across temperatures, with female responsiveness peaking near the average male IPI at different ambient temperatures. Further studies of the relationship between male courtship signals, direct and indirect benefits, and female preference functions in other firefly species will provide additional insight into the evolution of complex signaling systems.

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CALLS OF WILSON'S STORM PETREL: FUNCTIONS, INDIVIDUAL AND SEXUAL RECOGNITIONS, AND GEOGRAPHIC VARIATION

by

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(With 2 Figures)
(Acc. 31-III-1989)

Introduction

Most of the burrowing petrels are nocturnal on their breeding colonies, and the vocal activity appears therefore to be of prime importance for reproduction (STOREY, 1984) and pair-establishment (JAMES, 1985). Visual signals are thought to be secondary compared to vocal ones, especially in mate attraction and burrow defence (BROOKE, 1986). Mutual displays are entirely vocal, though a possible use of olfactory signals cannot be rejected (GRUBB, 1974). The functions of mutual displays have been summarized as i) advertisement, ii) synchronization of partners for breeding, and iii) species and sex recognition (HUNT, 1980; JOUVENTIN, 1972). It is presumed that these three functions are to be found the vocal activity of petrels. Another, but underemphasized feature of petrel breeding biology, is individual recognition, which has been tested experimentally (BROOKE, 1978; unpubl. data), and demonstrated by banding programs to be crucial to breeding success (GUILLOTIN & JOUVENTIN, 1980). The aim of this paper is to analyse the calls of Wilson's storm petrel *Oceanites oceanicus*, investigating their functions in advertisement, sexual and individual recognitions. Geographic

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variation in the calls and its possible consequences for species specific recognition are also investigated.

The family Oceanitidae (or Hydrobatidae) comprises the smallest birds of the Order Procellariiformes, with body length ranging from 14 to 26 cm. All the 20 commonly recognised species (HARRISON, 1983) are strictly pelagic marine birds and usually breed on islands. Only two species belong to the genus *Oceanites: O. gracilis* breeds on the Galapagos Islands, and is poorly known (HARRIS, 1969). In contrast, *O. oceanicus*, the Wilson's storm petrel, is widely distributed, and very numerous (several million pairs). Two subspecies are currently recognized, *O. o. oceanicus* breeding north of the Antarctic convergence, and *O. o. exaspeartus*, breeding south of it (BECK & BROWN, 1972). Although many studies have been carried out on its ecology, in the Antarctic (LACAN, 1971), on the Antarctic peninsula (ROBERTS, 1940) and on sub-Antarctic islands (BECK & BROWN, 1972; COPESTAKE & CROXAL, 1985), none of these however have dealt with the behaviour of this abundant species (for example, no sonograms have ever been published). In this paper, I provide a complete repertoire of Wilson's storm petrel vocalizations, followed by the function of each, as tested from the responses of the birds themselves. Individual and sexual recognitions were also investigated, by the analysis of 490 calls from 101 different individuals. Geographic variation in the calls is lastly noted, with tentative explanation for such differences.

Study areas and methods

The Pointe Géologie archipelago lies on the edge of Adélie land on the Antarctic continent, at 66°39′S, and 140°01′E. It constitutes more than 40 islets, only seven of which are large enough to support a breeding population of petrels. At Pointe Géologie, the Wilson's storm petrel breeds usually on slopes, using natural crevices in the rocks as nest chambers. Approximately 2000 pairs breed there (THOMAS, 1986), in dense colonies. Although there is no night in summertime, it is considered to be "nocturnal" in its activities at the colony (BRETAGNOLLE, 1988): birds begin to be active around 18 h local time, and leave the colonies early in the morning from 3 to 7 h. Field work was carried out on Pointe Géologie over a complete breeding season from December 1984 to February 1986. A second study site was used on the Kerguelen islands, situated between 48°27′S and 50°S in cold sub-Antarctic waters. Wilson's storm petrels are distributed in loose colonies throughout the islands, at altitudes below 800 m. Birds are active there during the night, between 22 h and 3 h local time, though first arrivals to the colony may begin at 18 h. Field work was carried out on these islands from November 1987 to February 1988.

Recordings were made using a NAGRA III B tape recorder and a Sennheiser omnidirectional microphone. The birds were often recorded from inside their burrows and sometimes when calling from outside, when the microphone was always less than 0.5 m away from the calling bird. The calls were analysed with a Kay 8800 Sound Spec-

trographic Display, and sonagrams were obtained using a Kay 6061 B Sound Spectrograph. The physical characteristics taken into account are shown in Fig. 1.

Playback tests were done in the field with a sound amplificator (10W) and two speakers (4W each). In a first set of experiments, the speakers were settled on two small promontaries (50 m apart from each other) in the colony, and all the birds flying or landing in a $2 \times 2 \times 2$ m^3 volume around the speaker were noted. The responses taken into account included: the number of birds flying directly over the speaker (''Direct flight''); the number of birds showing interest by flying in loops over the speaker (''Circle flight''); the number of birds landing (''Landed bird''). Only the two latter responses were considered as positive responses. In a second set, one speaker was placed at the entrance of an occupied burrow, and the sex and type of call made by the responding birds were noted.

Results

1. Description of vocalizations.

Grating call.

This vocalization has previously been described under several names: harsh chattering call (Roberts, 1940), nest advertisement call (Beck & Brown, 1972). Considering its physical characteristics, we prefer to name it grating call, keeping the ''chatter'' sound for another call. This was the commonest vocalization of the Wilson's storm petrel, and was used by both sexes. It was generally given from inside the burrow, though sometimes a single bird (most frequently a male) would utter it from a promontary, and exceptionally when flying. The grating call is constituted of syllables (Fig. 1) varying in number from three to over 40. Two variants of the call could be distinguished: a short call comprised three to six syllables, and a long one usually eight to 12 syllables but sometimes more. Syllables were very similar one another, whether considering calls of a single bird or of the whole population (Fig. 1).

Chattering call.

Although rather common, this vocalization has not previously been mentioned or considered only as an occasional variant of the grating call (Beck & Brown, 1972). It is constituted of a varying number of syllables but fewer than in the grating call. Unlike the grating call, the chattering call was given only by males. It was uttered preferentially from outside the burrow (from a promontary), though sometimes from within. In the latter case, mates were usually together and the chattering call was given in association with the grating call. It was never heard from a flying bird.

Other calls.

Two uncommon vocalizations complete the vocal repertory of the species; they both consist of repetition of a single syllable (Fig. 1): the peeping call (Roberts, 1940) appeared to be uttered by both sexes, especially when handled by man. In the few instances when it was heard under natural conditions, it was produced by flying or birds on the ground. The second call is structurally similar to the former, but lower in frequency. Rarely uttered, it could be the equivalent of the sparrow-like call described by Roberts (1940) and/or the call given at sea (Murphy, 1936).

Chicks produced only one vocalization, very similar to the peeping call of adults (Fig. 1). The chick call was emitted while a parent is in the burrow, and seemed to play the role of an appeasement contact call. It was possible to induce this call by touching the very young chick.

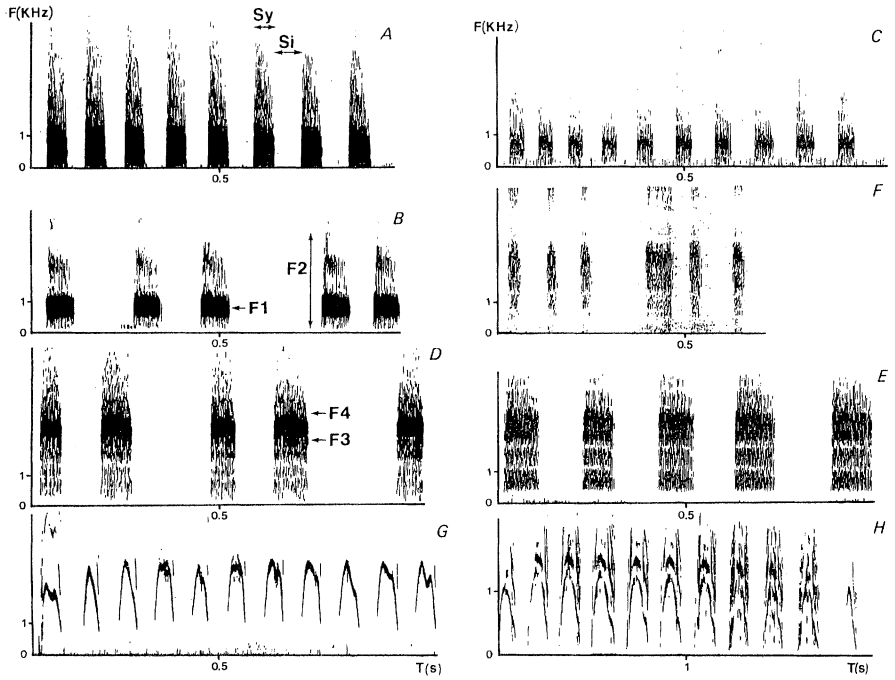

Fig. 1. Sonograms of Wilson's storm petrel vocalizations. Frequency in kHz and time in seconds. Sy and Si denote duration of syllable and silence respectively. F1 to F4 show frequencies used for analyses. A, B and C: chattering calls (A and B from Kerguelen, types B and A respectively (see text); C from Adélie Land). D, E and F: grating calls (D and E: male grating calls from Kerguelen and Adélie Land respectively; F: female grating call). G: chick call. H: peeping call of adult.

2. Experiments and numberings.

Experiments were conducted on the grating and chattering calls made by birds on Pointe Géologie. Table 1 summarizes the results of the first set of experiments with speakers placed on promontaries. The total number of responding birds shows that the chattering call was more attractive than the grating call (Table 1, last column; $t = 4.25$; $P < 0.001$). Significant differences appear between types of response given by birds while grating or chattering calls are played back ($t = 12.7$; $P < 0.0001$ for "circuit flight"; $t = 3.27$; $P < 0.01$ for "landing"). Even chattering and grating calls emitted together are more attractive than the grating call alone ($t = 3.42$; $P < 0.01$), but less than the chattering call alone ($t = 7.71$; $P < 0.001$). Lastly, no significant difference appears when no call or

TABLE 1. Numbers of Wilson's storm petrels responding to the playback of different calls

Played back call	Behavioural responses			
	Direct flight	Circle flight	Landed	Total
Chattering call n = 43	**0.74** (0-4)	**4.02** (1-6)	**1.16** (0-4)	**5.92**
	1.07	1.47	1.15	2.69
Grating call n = 43	**1.48** (0-10)	**0.7** (0-3)	**0.48** (0-3)	**2.66**
	2.23	0.88	0.73	3.88
Chattering and grating calls	**0.45** (0-2)	**1.4** (0-3)	**0.75** (0-2)	**2.6**
n = 20	0.76	1.14	0.63	2.53
No call played back n = 20	**2.05** (1-4)	**0.3** (0-2)	**0.2** (0-1)	**2.55**
	1.1	0.66	0.41	2.17

Mean values in bold, range in brackets and standard deviations below. These data were obtained in 20 days of experimentation.

grating call are played back ($t = 1.8$ for "circuit flight"; $t = 0.2$ for "landing").

The direct flight response (first column of Table 1) shows no significant difference for the three types of calls played back. However, chattering call, and chattering and grating calls both show significant differences to the control ($t = 5.35$; $P < 0.001$ and $t = 5.25$; $P < 0.001$). This rather surprising result (as "direct flight" has not been considered as a positive response) simply results from the presence of a "pool" of directly flying birds over the speaker (total of the row "control": 2.55 birds). When the chattering call is played back, some of the birds from the pool fly in circles or land, leaving less birds in "direct flight", and leading to significative differences.

In order to establish the status of attracted birds, storm petrels were mist-netted both while the chattering call was being broadcast and when it was not. Unfortunately, in the storm petrels so far studied, it is not possible to separate the sexes accurately (COPESTAKE *et al.*, 1988; FURNESS & BAILLIE, 1981). Only breeding females could be distinguished from other birds, by cloacal investigation (SERVENTY, 1956). Although fewer breeding females were caught when the chattering call was played back (Table 2), the difference is not significant ($P = 0.2$, Fisher exact test). However, working on the British storm petrel *Hydrobates pelagicus*, FURNESS & BAILLIE (1981) have shown that tape luring (with the purr-call, a highly attractive call) leeds to the capture of more wandering non-breeders than breeding birds. It is not unlikely that the same result should apply to Wilson's storm petrel.

TABLE 2. Status of mist-netted birds when a chattering call is played back or not

	Mist netted birds		Totals
	Breeding females	Males and non-breeding birds	
No call played back	**38** (47.5%)	**42** (52.5%)	**80**
Chattering call played back	**20** (38.5%)	**32** (61.5%)	**52**
Totals	**58**	**74**	**132**

TABLE 3. Frequency of responses by breeding birds to playback of different calls

Call played back	Male response			Female response			χ^2 Test
	Chattering	Chattering and grating	Grating	No call	Grating	No call	
Chattering (n = 39)	**0**	**0**	**15**	**8**	**6**	**10**	2.91 NS
Male grating (n = 39)	**0**	**0**	**12**	**7**	**4**	**16**	5.8 $P<0.01$
Female grating (n = 34)	**0**	**0**	**0**	**16**	**4**	**14**	2.17 NS

Differences between male and female responses are tested by χ^2 test.

Both circuit flying and landing were observed in response to the playback of the chattering call. On one night, we caught and banded nine landed birds with colour plastic rings. On the following days, we examined systematically all the accessible nests in the vicinity (within a radius of 25 m around the capture point) and re-discovered five birds. All were males, occupying a burrow less than 5 m away from the capture place. Similarly, we followed six birds in circuit flight over a calling male, and these were all females.

Although the numbers of birds involved in mist netting and these two observations are too small to permit definitive conclusions, they strongly suggest that birds respond differently to the chattering call according to their sex and reproductive status.

The responses of brooding birds to different types of played back calls are given in Table 3. Males only responded with grating calls to played back male calls (both grating and chattering). They also responded dif-

TABLE 4. Frequencies of types of call uttered by males in three different situations

Situation of the bird	Call uttered			χ^2 Test
	Chattering	Chattering and grating	Grating	
Bird alone (n = 96)	38	35	23	2.85 NS
Flown over by another bird (n = 69)	33	18	18	41.5 P<0.001
Facing another bird (n = 95)	9	16	70	48.7 P<0.001

A χ^2 test compares the results between first and second, second and third, and first and third situation, respectively.

ferently to male and female grating calls, thus indicating an ability to identify the sex of the calling bird ($\chi^2 = 12.7$; $P<0.001$). Males and females also responded differently to male grating calls ($\chi^2 = 5.8$; $P<0.01$).

Table 4 compares the types of calls given by males (from outside the burrow) in three situations: when alone, when flown over and when facing another bird. It shows that the grating call was essentially used during face to face interactions, while the chattering call was mainly given when the bird is alone.

Finally in Table 5, frequencies of the two types of male grating calls are compared when the interactions occur either between two males or between a male and a female. The long version was used in agonistic interactions, while the short one served principally in sexual interactions.

3. Sexual differences in calls.

We have shown (Table 3) that birds do recognize the sex of the calling bird. The chattering call, which was only given by males, thus has a potential role in sexual recognition. However, the grating call which was performed by both sexes, had different temporal and frequency characteristics for each sex (Table 6). Female grating calls had a faster tempo and their tone sounded clearer (pers. obs.) and higher than male grating calls (Fig. 1). It seems then that the grating call serves a role in sexual recognition by Wilson's storm petrels.

THE ROYAL SOCIETY

Response of foraging group members to sentinel calls in suricates, *Suricata suricatta*

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In the suricate (*Suricata suricatta*), a cooperatively breeding mongoose, one individual typically watches out for predators while the rest of the group is foraging. Most of the time these sentinels announce their guarding duty with special vocalizations. The response of foraging group members to these calls was investigated by analysing observational data, and by performing playback experiments. The use of special calls by sentinels, and the responses of the foraging group members to them, suggest that the coordination of vigilance is strongly influenced by vocal communication. Sentinel calls decreased the time spent alert by the foraging group members. Other group members were less likely to go on guard when a sentinel was vocalizing. Both the proportion of time during which guards overlapped, and the proportion of time the group was unprotected without a guard, decreased when sentinels announced their duty, due to better coordination of the rotation of sentinels. Suricates, however, do not appear to use sentinel calls to mediate a strict rotation of guarding duty.

Keywords: sentinel calls; sentinels; vigilance; cooperative breeding; suricates

1. INTRODUCTION

Vigilance behaviour in some social bird and mammal species is coordinated by sentinel systems, in which individuals take turns in watching for predators, while the rest of the group is foraging (for a review, see Bednekoff 1997). It has been suggested that such behaviour allows each foraging member to decrease its own vigilance and gain additional time to search for food (McGowan & Woolfenden 1989; Bednekoff 1997). However, Ward (1985) pointed out that in bird flocks the costs of coordinating vigilance exceeded the potential benefits. He argued that birds have to interrupt their foraging to check visually for a guard on duty. Wickler (1985) observed that in various babbler species, coordination of vigilance may be independent of visual scanning because guards announced that they were on duty using a particular vocalization, the 'watchman's song'. Bednekoff (1997) suggested that while the vocalization of guards might make sentinel systems run more smoothly, it is not a necessary condition for the evolution of sentinel behaviour. Goodwin's definition (Goodwin 1976; McGowan & Woolfenden 1989) of true sentinel behaviour requires interaction between the guards and those being protected; vigilant individuals guard others and alert them to danger, and the individuals take turns in guarding. Therefore, special sentinel calls to announce vigilance may not be essential for a sentinel system, but they might increase its efficiency. This may become especially important in animals living in habitats which do not permit a clear view of the sentinel, because vocal announcement of guarding would eliminate the necessity for foraging members to check visually for a sentinel (Metcalfe 1984; Rasa 1986). Vocalizations to announce guarding duties have also been described in other species including the Florida scrub jay, *Aphelocoma coerulescens* (McGowan & Woolfenden 1989), the white-browed sparrow weaver, *Plocepasser mahali* (Ferguson 1987), the dwarf mongoose, *Helogale parvula* (Rasa 1986) and the suricate, *Suricata suricatta* (Moran 1984; Macdonald 1992; Doolan 1997).

Wickler (1985) described watchman's calls in several babbler species as being calls of short duration and soft amplitude that were emitted continuously over the whole guarding period. Dwarf mongooses also announce their guarding continuously by emitting a loud version of contact calls (Rasa 1986). For other species, such as the white-browed sparrow weaver (Ferguson 1987), or the Florida scrub jay (McGowan & Woolfenden 1989), calls of sentinels are only given infrequently at the beginning or end of a guarding session, but nevertheless have been suggested to have some function announcing sentinel duty.

Vocal announcement of a sentinel on duty may not only allow the foraging members to decrease their vigilance, but could also help to coordinate guarding behaviour between the group members. In dwarf mongooses, group members rotate their guarding in a regular pattern in which each individual takes over from a particular member of the group (Rasa 1986). Rasa suggested sentinel calls informed the foraging individuals about the guard's identity and location, and further enabled the sentinels to rotate without overlapping their vigilance periods.

Suricates are cooperatively breeding mongooses living in open semi-desert areas in packs of between three and 25 members, consisting of adults, subadults and dependent young (Doolan 1996*a*,*b*; Clutton-Brock *et al.*

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1998*a*). A group forages together, and they often have a sentinel watching out for predators (Moran 1984; Doolan 1997). On the majority of occasions, sentinels announce their guarding vocally, but sometimes remain silent (Macdonald 1992; Doolan & Macdonald 1997). Suricates dig in the sand for food, and regularly interrupt their foraging to scan for predators, or to check if another individual is on guard. When a sentinel is announcing its duty, the foraging members may decrease their vigilance and hence interrupt their foraging less frequently. In a species that digs for mobile prey in the ground, this could substantially increase foraging success.

In this paper, I describe the vocalizations of sentinels in suricates and their effect upon foraging group members. First, I show that the sentinel system in suricates is effective in detecting predators. I then describe sentinel vocalizations and examine whether these calls are emitted continuously over the whole guarding period. Furthermore, I investigate the response of foraging members to the calls, and ask (i) do foraging members decrease their own vigilance when they hear sentinel calls? (ii) are foraging individuals less likely to go on guard in response to sentinel calls? (iii) does the coordination of guarding improve? and (iv) do suricates use sentinel calls to mediate the rotation of sentinel duty?

2. METHODS

Data on sentinel vocalizations were collected from six different groups in the Kalahari Gemsbok National Park, South Africa, along the dry Nossob river-bed (Clutton-Brock *et al.* 1998*b*), from a total of 42 different individuals. For most of the analysis, however, sufficient data were only available from four groups. Group size varied from four to 20 members in total, including dependent offspring. The members of the groups could be recognized individually and were habituated to the point where I could walk with them and make recordings from 50 cm. Calls were recorded using a Sony^{TM} digital audio tape recorder DAT Pro II TCD D10 and a Sennheiser^{TM} directional microphone MKH 816. Playbacks were performed with a Sony^{TM} DAT recorder connected to a Sony^{TM} Walkman loudspeaker SRS A60. Spectrograms were analysed with the Kay Sonagraph Analyser, Model 5500, using a 150 Hz wide band set-up (Kay Elemetrics Corp. 1989, Pine Brook, NJ, USA).

(a) *Sentinel behaviour*

To determine whether sentinel calls have a vigilance function I first investigated whether sentinels in suricates followed the definition proposed by Goodwin (1976), by warning other individuals of danger. The alarm call frequency of individuals when foraging and when guarding were compared. Any suricate going on raised guard on an elevation higher than 10 cm was considered as a sentinel, while any other individual was taken as foraging. Whenever an alarm call was given the identity of the caller was recorded as well as its behaviour, categorized either as sentinel duty or foraging.

(b) *Sentinel vocalization*

Whenever an individual went on raised guard, its identification, the duration of the guarding period, and whether it had been vocalizing at any stage (if possible) were recorded. I recorded the types of vocalization emitted over the entire guarding period for a total of 60 guarding sessions of 24 different individuals. This enabled me to compare the calls used by different individuals and the change in the calling pattern over time. Furthermore, I analysed sonograms of the calls of 12 individuals from four different groups to compare the structure of calls used by different individuals within and between groups.

(c) *Response of foraging members to sentinel vocalization*

(i) *Vigilance of foraging members*

The response of foraging members to sentinel calls was investigated by analysing observation data, and by performing playback experiments. I tested whether the time foraging individuals spent alert decreased when a sentinel was vocalizing, and also how frequently they went on guard during periods when a sentinel was vocalizing in comparison with when it was quiet. The influence of sentinel vocalizations on the rest of the group was also tested by playing back the calls to a foraging individual when there was no sentinel. The vocalization of a guarding individual was recorded and then broadcast to foraging suricates in the same group ($n = 4$ groups), following an individual at a distance of 3–5 m with a small Walkman loudspeaker at a height of 80 cm. The behaviour of the target suricate was recorded on a PSION^{TM} datalogger, Organizer II, Model L264, concentrating on the time spent foraging and alert (including looking up, guarding on the ground and raised guard). Two control sessions of half an hour each were conducted: one in which background noise (recorded in the field without disturbances by birdsong or other animals calling) was played, and the other in which contact calls of a member of the same group were played. This was repeated once for all the different test periods, giving a total of a 1 h experiment and a 1 h control session. To avoid an order effect, the experiment and the control periods were conducted alternately. If, during the test or control period, another member of the group went on guard (raised guard or guard on ground with vocalization), the data recording was interrupted to make sure that the vocalization was responsible for the effect, and not the view of a sentinel.

(ii) *Probability of going on raised guard*

To test whether having a guard and hearing sentinel vocalizations decreased the frequency of other individuals going on raised guard, I compared observational data from four groups for three different periods: (i) when no sentinel was up, (ii) when a sentinel was up but not vocalizing, and (iii) when a sentinel was up and emitting the guarding calls. In the first case, with no sentinel on guard, I calculated the average time it took for an individual to go on guard since the last sentinel had left its position. For the other two periods, when a sentinel was up without vocalizing or with vocalizing, I calculated the time from when a sentinel had taken its position until the next individual went on guard. From the 12 playback experiments, it was also possible to estimate the average time between guards when the suricates heard the sentinel vocalization, but did not see a guard up.

(iii) *Probability of terminating raised guard*

Observational data were also used to investigate whether the probability of a guarding session terminating depended on whether a sentinel was vocalizing. I measured the duration of overlapping guards, documenting the time which lapsed between a second individual going on guard and the first sentinel leaving its position, and resuming foraging again. In

addition, I tested whether vocalizations from either the first or second sentinel influenced which individual stayed on guard.

(iv) *Coordination of guarding*

I was then able to estimate the influence of vocal announcement of sentinel duty on the coordination of guarding behaviour and the increase in foraging time from observational data. I compared foraging periods when sentinels vocally announced their duty and when they kept silent, by looking at the amount of time that (i) one sentinel was up, (ii) there was more than one sentinel, and (iii) no sentinel was up. Foraging periods coordinated by sentinels vocalizing included all sessions when one or several sentinels were on duty and at least one of them calling. In addition, the periods after a vocalizing sentinel had left, but no other individual was on guard, were summed, and the percentage of the whole period covered by a sentinel calculated. Whenever a sentinel went on guard without calling it was counted as a period of silent sentinels, as was the time after that guarding session until another individual went on guard and announced its duty again. This was done for all the observation periods per group, and the mean time was standardized to 1 h foraging time.

(b) *Rotation of guarding*

To investigate whether suricates use sentinel calls to coordinate the rotation of guarding, I tested whether certain individuals went on guard predominantly after a specific individual. In four groups I analysed the sequence of the different individuals in a group going on sentinel duty.

(c) *Statistics*

Almost always non-parametric tests were used because the sample sizes were small (Zar 1984). U indicates a Mann–Whitney test, Z a Wilcoxon signed-rank test, H a Kruskal–Wallis test and a chi-square or a Friedman test. In some comparisons, such as the probability of going on raised guard, I performed a Mann–Whitney test by using the group as an independent data point ($n = 4,4$), rather than conducting a Wilcoxon test on the level of each experiment, since this would have included repeated measures on the same group. In my analysis testing the influence of sentinels vocalizing and the duration of guard on the probability of terminating sentinel behaviour, I performed an ANOVA with repeated measures and quote the F-value. All p-values given are two-tailed. The error bars in the figures show inter-quartile ranges, and the circles denote the ranges.

3. RESULTS

(a) *Sentinel behaviour*

Sentinels in suricates appear to look out for predators and warn other group members in case of danger by alarm calling. The same individual on sentinel duty gave alarm calls more often than when it was foraging (alarm call rate on sentinel duty: 0.86 ± 0.13 calls h^{-1}; while foraging: 0.08 ± 0.01 calls h^{-1}; Wilcoxon test: $Z = -5.704$, $n = 48$, $p < 0.001$).

Furthermore, the efficiency of predator detection improved when a sentinel was up. Suricates acting as sentinels called alarms to potential predators in 77% out of all predator detections by sentinels when they were more than 150 m away, compared with foraging individuals which only emitted 44% out of all their alarm calls to predators at this great distance (chi-square: $\chi^2 = 10.31$, d.f. $= 1$, $p = 0.0013$).

(b) *Sentinel vocalization*

Suricate sentinels announced that they were on duty with special vocalizations. For 55% of the time foraging suricates had a guard on an exposed lookout. Guarding individuals spent 80% of their time vocalizing. In total, six different calls, in addition to alarm calls, were emitted by sentinels (figure 1). The four most frequently given sentinel calls ($> 95\%$ of all emitted sentinel calls) consisted of a single or multiple note of short duration and these calls were only audible to the human ear up to a distance of 15–20 m. The two less common sentinel calls were of longer duration and might function to alert other individuals. However, this has still to be tested. The same vocalizations ($n = 4$: single, double, triple and multiple note) significantly differed between individuals in duration (Kruskal–Wallis: $H = 35.55$, d.f. $= 11$, $p = 0.0002$) and fundamental frequency (Kruskal–Wallis: $H = 35.73$, d.f. $= 11$, $p = 0.0002$), and seemed not to be a group characteristic (table 1). Each member had its own call characteristic, having both a consistent call duration (Friedman test: $\chi^2 = 1.41$, d.f. $= 2$, $p = 0.49$) and fundamental frequency (Friedman test: $\chi^2 = 0.18$, d.f. $= 2$, $p = 0.91$) in their three most frequently emitted guarding calls (single, double and triple note call, 81% of all sentinel calls observed).

Sentinels changed their calling frequency over a guarding period. Individuals began to vocalize from the beginning of a period. The number of calls given in the first two minutes of acoustically announced guards was on average 19.8 ± 14.3 ($n = 24$ individuals). Vocalization ceased sometime before the individual left its guarding position (Friedman test: $\chi^2 = 27.7$, d.f. $= 4$, $p < 0.0001$, $n = 24$ individuals) (figure 2). No terminal call was observed. In most cases the individual leaving its guarding position immediately gave contact calls, either while descending or as soon as it began to forage again.

(c) *The influence of sentinel calls on the foraging members*

(i) *Vigilance of foraging members*

Playing back sentinel calls to foraging members reduced their alertness. In 12 experiments, in which sentinel calls were played to a foraging member when there was no individual on guard, the time the target individual spent vigilant substantially decreased compared with the control period (Wilcoxon: $Z = -3.06$, $n = 12$, $p = 0.002$) (figure 3). In the additional control experiment when the influence of contact calls was tested on foraging individuals, the proportion of time spent vigilant did not differ markedly between the control and experimental sessions (Wilcoxon: $Z = -0.52$, $n = 6$, $p = 0.60$).

(ii) *Probability of going on raised guard*

At times when there was already a sentinel up, the other members in the group were less likely to go on raised guard. The analysis of the time interval between guards in four groups showed a significant difference for the three different conditions, when there was (i) no sentinel, (ii) a quiet sentinel, and (iii) a sentinel vocalizing.

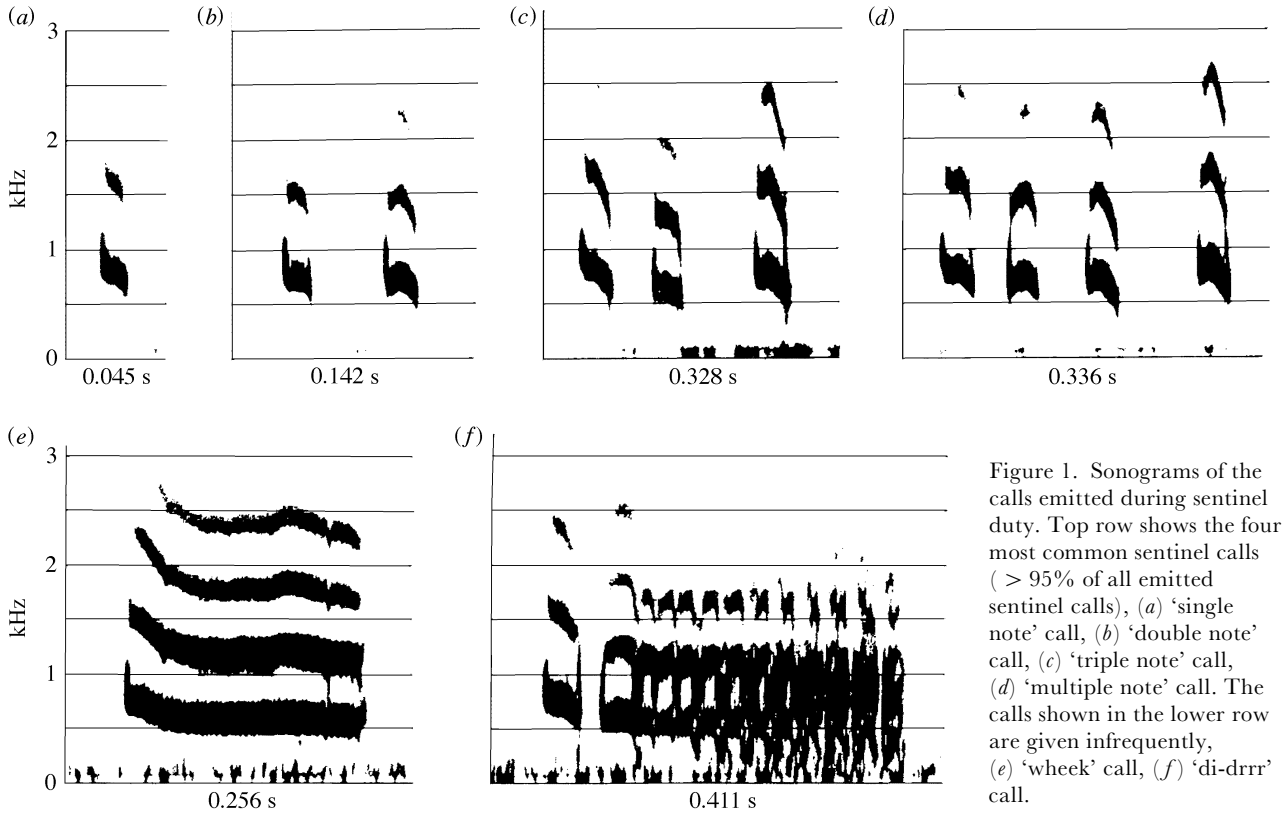

Figure 1. Sonograms of the calls emitted during sentinel duty. Top row shows the four most common sentinel calls (> 95% of all emitted sentinel calls), (*a*) 'single note' call, (*b*) 'double note' call, (*c*) 'triple note' call, (*d*) 'multiple note' call. The calls shown in the lower row are given infrequently, (*e*) 'wheek' call, (*f*) 'di-drrr' call.

Table 1. *Fundamental frequency and duration (mean) of the most frequently used sentinel calls for several individuals with different status and sex from four groups*

(sn, single note call; dn, double note call; tn, triple note call; mn, multiple note call with greater than three notes per call; sub, subordinate; dom, dominant. In the case of the double and triple note call, only the fundamental frequency and duration of the first note were considered.)

			fundamental frequency (Hz)				duration of first note (ms)			
sex	status	group	sn	dn	tn	mn	sn	dn	tn	mn
male	sub	A	1040	992	960	960	26.7	28.4	26.0	28.5
male	dom	A	792	792	808	800	59.2	55.8	55.6	58.0
male	sub	A	1000	1010	1000	1020	39.6	40.4	40.8	42.2
female	dom	J	1010	960	1040	1020	43.2	45.5	42.0	48.1
male	dom	J	768	760	760	820	45.4	47.3	42.0	44.0
female	sub	J	1100	1112	1120	—	43.0	44.6	41.5	—
male	sub	J	1120	—	—	—	59.0	—	—	—
female	sub	N	980	1000	920	—	36.0	41.0	43.0	—
female	sub	N	780	770	807	840	34.3	30.6	32.0	39.0
male	dom	N	740	744	752	—	46.0	46.2	46.0	—
female	dom	S	880	887	880	880	53.0	52.3	54.0	56.0
male	sub	S	1080	1144	1107	—	28.3	30.8	32.7	—
female	sub	S	800	773	—	—	42.0	43.0	—	—

The time interval until the next individual went on raised guard rose substantially when there was already a sentinel up, and increased even more when the sentinel was emitting calls (Kruskal–Wallis: $H_{4,4,4} = 8.77$, $p = 0.013$, $n = 4$ groups) (figure 4*a*).

The longer interval between individuals going on guard when a sentinel was vocalizing than when it kept silent, could be explained by the fact that announced guards in general tended to be longer than guards where individuals did not emit sentinel calls (Manser *et al.* 1999). However, the playback experiments of sentinel calls confirmed the result in that the time between guards increased from the control period with background noise to the test period with sentinel calls (Mann–Whitney: $U = 16.0$, $p = 0.03$, $n = 4,4$ groups) (figure 4*b*). The experiment was conducted three times in each of the four different groups, and the mean value per group was used as the independent data point.

The interval between guards when no sentinel was up was shorter during the observational periods than

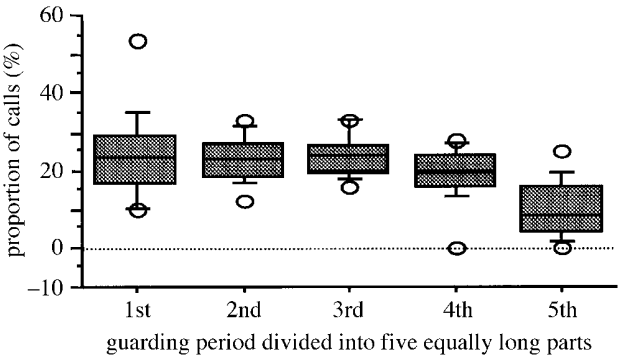

Figure 2. Proportion (per cent) of sentinel calls given in the different parts of a guarding session. The box plots show 10th, 25th, 50th, 75th and 90th percentiles. The circles denote ranges.

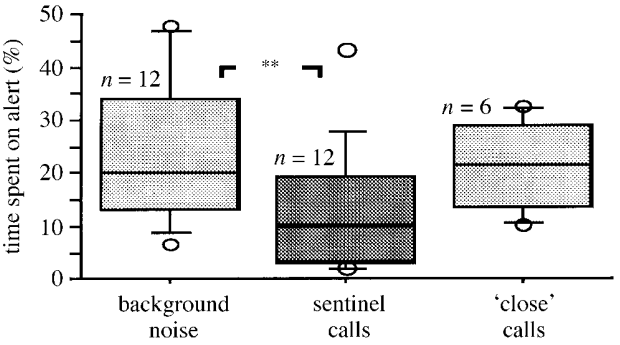

Figure 3. Time spent alert per hour of foraging period in the control and test periods. The box plots show 10th, 25th, 50th, 75th and 90th percentiles. The circles denote ranges (** = $0.01 > p > 0.001$).

in the playbacks. This apparent discrepancy arose because experiments were conducted at times when individuals were not going on raised guard very often, so that it would be clear that group members were responding to the playback, and not to the sight of a sentinel on duty.

(iii) *Probability of terminating raised guard*

The vocalizations of sentinels were associated with the probability that they would stop guarding. When another individual went on guard, sentinels emitting calls did not terminate their guarding as fast as non-vocalizing sentinels. The average time a sentinel stayed on guard when another individual went up was always longer for vocalizing sentinels than silent sentinels, independent of the duration a sentinel had already been up (ANOVA with repeated measures: $F_{1,20} = 67.36$, $p = 0.0004$).

Whether a sentinel already up left its position when a second individual went on guard depended less on whether the first sentinel was vocalizing than on whether the second sentinel began to emit calls. If the second sentinel was silent, it stopped guarding before the first sentinel in 74.3% of cases (total $n = 316$ guarding periods). If the second guard vocalized, on the other hand, it terminated its guard before the first sentinel in only 32.6% of cases (chi-square: $\chi^2 = 53.35$, d.f. = 1, $p < 0.0001$). This was independent of whether the first sentinel was vocalizing (chi-square: $\chi^2 = 0.52$, d.f. = 1, $p = 0.47$).

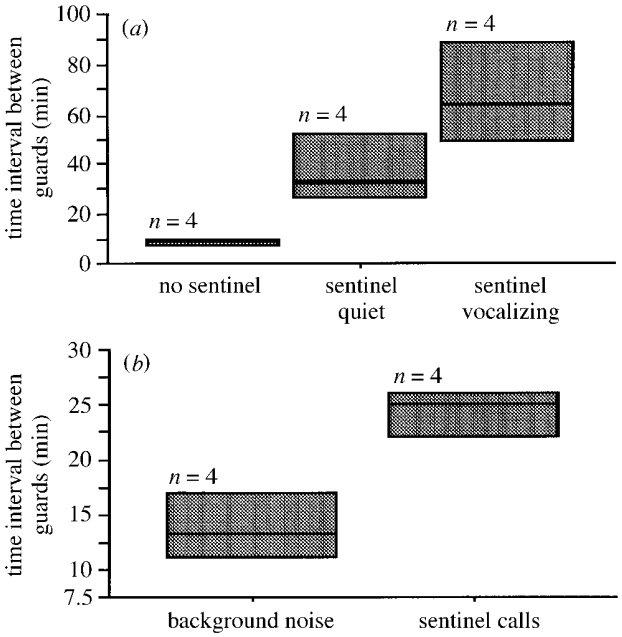

Figure 4. (*a*) Time interval between guards during foraging depending on whether there was (i) no sentinel up, (ii) a sentinel without vocalizing, and (iii) a sentinel emitting calls. The box plots show 25th, 50th and 75th percentiles. (*b*) Time interval between guards when playbacks of (i) background noise, and (ii) sentinel calls were broadcast. The box plots show 25th, 50th and 75th percentiles.

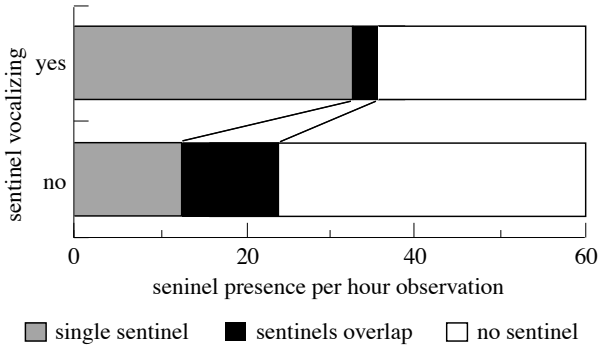

Figure 5. Proportion (median) of protected and unprotected time per hour of foraging when the sentinel was vocalizing or not.

(iv) *Coordination of guarding*

The vocalizations of a sentinel influenced the coordination of guarding behaviour within the group (figure 5). The mean duration of guarding periods was longer if the sentinel vocalized (duration: median = 8.42 min, interquartile range (IQR) = 1.89), than if it did not (duration: median = 3.4 min, IQR = 1.13; Mann–Whitney: $U = 16.0$, $n = 4,4$, $p = 0.02$). The unprotected time between guarding attempts decreased slightly, but not significantly if the previous sentinel had been vocalizing (silent: median = 12.9 min, IQR = 4.56; vocalizing: median = 8.7 min, IQR = 1.73; Mann–Whitney: $U = 10.0$, $p = 0.56$, $n = 4$ groups). Even though the overlapping time per guarding attempt was longer when the sentinel was vocalizing (median = 3.28 min, IQR = 1.45) compared with when it was quiet (median = 2.62 min, IQR = 1.83), there was a

shorter relative overlap when vocalizing, because the guard duration was more than 2.5 times longer for a vocalizing sentinel than for a quiet sentinel. Whether a sentinel emitted calls or not, an immediate turnover of guarding in which there was no overlap between sentinels happened in about 10% of observed guarding periods. Guards overlapped in 30% of their guarding periods when the sentinel was quiet and in only 20% of guarding periods when the sentinel was announcing its duty (Mann–Whitney: $U=16.00$, $p=0.02$, $n=4$).

Overall, this resulted in better coordination during times when sentinels vocalized, as the total protected time for the foraging group increased (quiet: median $=23.82\,\mathrm{min\,h^{-1}}$, IQR $=6.52$; vocalizing: median $=36.18\,\mathrm{min\,h^{-1}}$, IQR $=6.52$; Mann–Whitney: $U=16.0$, $p=0.02$, $n=4$), and the overlap of sentinels decreased (quiet: median $=11.5\,\mathrm{min\,h^{-1}}$, IQR $=4.36$; vocalizing: median $=3.66\,\mathrm{min\,h^{-1}}$, IQR $=2.0$; Mann–Whitney: $U=16.0$, $p=0.02$, $n=4$) as shown in figure 5 for a 1 h foraging period.

(d) *Rotation in guarding behaviour*

In none of the four tested groups was a pattern found where certain individuals predominantly took over from a specific individual. A log-likelihood ratio analysis showed that there was no strong evidence of non-random associations (group 1: $G=19.2$, d.f. $=25$, $p=0.8$; group 2: $G=14.5$, d.f. $=9$, $p=0.1$; group 3: $G=26.3$, d.f. $=25$, $p=0.4$; group 4: $G=49.3$, d.f. $=81$, $p=0.99$).

4. DISCUSSION

The use of special vocalizations by sentinels, and the response to them by foraging members, suggest that the coordination of vigilance behaviour in suricates is strongly influenced by auditory communication. Sentinels in suricates announced most guards by special short, soft calls. Foraging individuals showed a significantly lower level of vigilance in response to playbacks of sentinel calls than to playbacks of background noise or contact calls. During periods in which sentinels announced their duty, the coordination of their guarding rotation also became more efficient. Suricates, however, do not use sentinel calls to mediate the rotation of guarding duty.

In suricates about 55% of foraging time was protected by having a sentinel on guard. During 80% of this guarding time the sentinels announced their duty vocally. When sentinels were vocalizing, not only did the coordination of the guarding system increase, but so did the time spent foraging by each individual, as they interrupted their activities less often to scan their surroundings. Thus, vocalizations improved the efficiency of the suricate sentinel system. On the other hand, the guarding bouts without vocalizations confirm Bednekoff's suggestion that vocalizations might help to run sentinel systems more smoothly, but they are not essential for sentinel systems to evolve (Bednekoff 1997). However, for a species such as the suricate, living in a harsh environment with limited food resources and high predation risk, a 10% increase of foraging time, and a likely higher rate of foraging success, because they do not have to interrupt foraging bouts to scan for predators, may have a large impact on reproduction and survival (Clutton-Brock *et al.* 1998*a*).

Although the vocalizations emitted by sentinels included information about their identity, suricates seemed not to use this information to mediate the rotation of their guarding. The four most frequently used sentinel calls were consistent for each individual in fundamental frequency and duration, but differed between group members. This would allow individual recognition by calls. Rasa (1986) suggested that the dwarf mongoose may use the recognition of individuals by calls to coordinate the rotation of the guarding period. No such regular pattern of an individual taking over predominantly from one particular individual was observed in suricates, and other factors, such as foraging success, might be more likely to influence the guarding rotation (Clutton-Brock *et al.* 1999; Manser *et al.* 1999). Instead of using the information about the identity of a guard to coordinate the rotation, they might use it to estimate the quality of a guard, e.g. reliable versus unreliable sentinels. This has yet to be tested.

An acoustically coordinated sentinel system, such as that in suricates, substantially increases foraging time for the group members when compared with a system where a guard must be visually located. The advantages seem especially obvious in social foraging groups moving in search of food on the ground, through habitat with poor visibility (Rasa 1986). Acoustical announcement of sentinel duty might be less important for pair-living species, such as klipspringers, *Oreotragus oreotragus* (Tilson 1980), where only the partner is guarding and its position is easy to localize. This would also apply to species foraging in open fields, as described for vervet monkeys, *Cercopithecus aethiops* (Horrocks & Hunte 1986). Benefits of vocalizing are also restricted to groups that forage close together, as they remain within hearing range.

The evolution of continuous announcement of sentinel duty offers a simple system to coordinate the vigilance behaviour among all individuals in a group. As long as foraging individuals hear sentinel calls they know somebody is guarding and can decrease their own vigilance. When they do not hear sentinel calls any more, either there is no guard or the current guard is about to leave its position and has to be replaced. It seems unlikely that a system where individuals just announce the beginning or the end of their guarding periods (McGowan & Woolfenden 1989) would be as efficient under such circumstances, as the chance of missing those signals, as a receiver moves through dense habitat, might be very high.

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The information that receivers extract from alarm calls in suricates

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Field observations and acoustic analyses have shown that suricate (*Suricata suricatta*) alarm calls vary in their acoustic structure depending on predator type. In this study, we tested whether receivers respond appropriately when hearing a call in the absence of a predator. Although the only way for suricates to escape from predators is to retreat to boltholes, responses to playbacks could be divided into distinct categories. The subjects responded differently to alarm calls given in response to aerial or terrestrial predators and to recruitment calls emitted in response to snakes and deposits on the ground. Suricates also showed rather distinct responses to low, medium and high urgency aerial calls. Differences in the responses were less obvious for different levels of urgency in the terrestrial and recruitment calls. Suricate receivers thus gain information about both the predator type and level of urgency from the acoustic structures of their calls.

Keywords: alarm calls; functionally referential; level of response urgency; receivers; suricates

1. INTRODUCTION

In order to understand the evolution of acoustic variation in animal vocalizations, it is important to consider both the contexts that elicit different calls by signallers and the information that receivers extract from these calls (Marler *et al*. 1992; Macedonia & Evans 1993). In the case of alarm calls, two different meanings that are extracted by receivers have been described. In some species, the acoustic structure of the alarm calls varies depending on predator type and this variation is sufficient to allow receivers to respond appropriately to different types of predators, even in the absence of visual cues. Such calls have been described as functionally referential. Vervet monkeys (*Cercopithecus aethiops*), for example, typically show obviously different responses to different types of alarm calls. They run into trees in the case of leopard alarm calls and to the next bush when hearing eagle alarm calls (Seyfarth *et al*. 1980). Several species of sciurids, such as some ground squirrels (Robinson 1981) and marmots (Blumstein & Armitage 1997*a*), also give more than one call type in response to different predators. However, their calls appear to be less predator specific and rather denote slow-developing 'low-risk' and fast-developing 'high-risk' situations', respectively (Robinson 1981; Sherman 1985). The only way of escape, independent of what call type is played, is to run into their burrow. The alarm calls of these species have therefore been assumed to provide listeners with information about the urgency of the situation rather than the predator type.

The selective force causing some species to evolve functionally referential alarm calls, as opposed to only response urgency alarm calls, might be the way of escape from a predator (Cheney & Seyfarth 1990; Macedonia & Evans 1993). When reviewing alarm calls in several primate and sciurid species, Macedonia & Evans (1993) concluded that the predation pressure on small-bodied, terrestrial mammals living in an open habitat might explain why vervets' and ring-tailed lemurs' (*Lemur catta*) alarm calls denote predator classes and possibly also urgency, while the alarm calls of arboreal-ruffed lemurs (*Varecia variegata*) appear to be threat and recruitment/mobbing calls (Macedonia 1990). However, this explanation does not explain why ground squirrels have evolved alarm calls that are urgency based but not functionally referential. Macedonia & Evans (1993) suggested that functionally referential-specific alarm calls are favoured by natural selection when animals confront different predator species with different hunting strategies where different modes of escape are advantageous (Marler 1967; Cheney & Seyfarth 1990; Macedonia & Evans 1993). According to this view, the degree of referential specificity of each alarm call and also the number of call types within a species' repertoire are determined by the function of the call. For example, because vervet monkeys confront a variety of different predators with different hunting strategies, they have evolved different escape strategies. Presumably as a result, the monkeys possess a number of acoustically distinct predator-specific alarm calls (Struhsaker 1967; Seyfarth *et al*. 1980). In contrast, predator type is less important for some sciurid species than the immediacy of danger, since they escape from any predator by fleeing to their burrows (Blumstein & Armitage 1997). The question arises as to whether only species with multiple ways of escape evolve functionally referential alarm calls or whether other forces can cause the evolution of highly sophisticated alarm systems.

Suricates (*Suricata suricatta*), also called meercats which are cooperatively breeding mongooses, use several structurally distinct alarm calls for warning other group members when predators are approaching. The acoustic structure of some of their alarm calls varies depending on both predator type and the level of urgency (Manser 2001). Suricates are diurnal and live in open semi-desert areas in groups of three to 33 individuals. They forage for 5–8 h d^{-1} in the open, digging for invertebrates and small vertebrates in the sand (Doolan & Macdonald 1996).

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Table 1. The number of playback experiments performed per call type (indicating what predator type and level of urgency the calls denote) in different groups.

call type	predator type	level of urgency	*n* different calls per call type	*n* groups	*n* playback experiments
aerial low urgent	aerial	low	6	6	6
aerial medium urgent	aerial	medium	12	12	18
aerial high urgent	aerial	high	6	6	12
terrestrial low urgent	terrestrial	low	6	6	6
terrestrial medium urgent	terrestrial	medium	6	6	12
terrestrial high urgent	terrestrial	high	6	6	6
recruitment low	recruitment	low	6	6	12
recruitment high	recruitment	high	6	6	12
alert	aerial	low	12	12	18
moving animal	aerial/terrestrial	low	12	10	18
panic	aerial/terrestrial	high	6	6	12

They spend most of their time foraging at a distance of 20–50 m from the next bolthole or shelter and, when passing by during foraging, they often renovate these holes. Suricates emit a variety of acoustically different calls in order to alert other individuals to the approach of aerial and terrestrial predators. Alarm-like calls are also used for recruiting group members when encountering snakes or other animals caught in boltholes or for recruiting others for investigating deposits on the ground, such as urine, faecal or hair samples of foreign suricates or predators (Manser 2001). Depending on the alarm call given, suricates either stand bipedally, run to the next bolthole or move to a burrow system. When they encounter a snake, they gather together and mob the snake. When they smell a deposit they begin to emit calls and other group members join them in order to investigate it. Although suricates frequently climb up shrubs and trees to stand guard, they never climb to escape from predators.

In this study, we use playback experiments in order to investigate how suricates respond to different alarm call types that vary in their acoustic structure depending on both the level of urgency and predator type. The range of alarm call types in suricates allows us to examine whether the variation in acoustic structure with the level of urgency elicits the same range of responses as does the variation in acoustic structure with predator type. In particular, we ask whether suricates show different responses to aerial and terrestrial alarm calls and recruitment calls and whether they respond more strongly to more urgent calls within the same predator category.

2. METHODS

(a) *Study site and animals*

Recordings of alarm calls and the playback experiments were conducted at two study sites in South Africa in the southern part of the Kalahari Desert from November 1995 to December 1996 and again from January 1999 to August 1999. For the recordings of alarm calls, we followed eight groups with 70 adult individuals along the dry riverbed of the Nossob in the Kalahari Gemsbok National Park and another 10 groups with 131 adult individuals on ranchland close to Van Zyl's Rus along the dry riverbed of the Kuruman (for a more detailed description see Clutton-Brock *et al*. (1998)). All individuals in a group could be identified and were habituated to close-range observation by humans on foot. We made tape recordings of vocalizations from as close as 0.2 m. Alarm calls were recorded using a Sony digital audio tape recorder DAT Pro II (Sony Corporation, Tokyo, Japan) and a Sennheiser directional microphone MKH 816 (Sennheiser electronic, Wedemark, Germany).

(b) *Playback experiments*

(i) *Selection of calls for playback experiments*

Acoustic analysis of the different alarm-call types conducted prior to the playback experiments showed that the acoustic structures of the calls varied substantially within a predator type, but also depending on the level of response urgency (Manser 2001). We investigated whether the receivers would recognize these different calls as being from the correct category of predator type and show the appropriate response, and also whether there was a difference in their responses depending on the level of urgency a call conveyed. We tested responses to 11 different alarm-call types that had originally been elicited by the presence of different predator types at different levels of response urgency (aerial and terrestrial calls at low, medium and high urgency levels and recruitment calls at low and high urgency levels) (table 1). We also played three other types of call that were not correlated with specific predator types, namely 'alert', 'moving animal' and 'panic' calls. The alert and moving animal calls were given in response to a variety of predators as well as non-dangerous animals, usually in what appeared to be low-risk situations. Panic calls, on the other hand, were elicited in response to the alarm calls given by birds and a few times by aerial and terrestrial predators that were very close by. These calls almost always made the suricates run to the next bolthole and go below ground, suggesting that they represented a high-risk situation. An example of a typical spectrogram and a description of the context that elicited the different call types are given in another paper on an acoustic analysis of these vocalizations (Manser 2001).

(ii) *Experimental procedure*

Responses to alarm calls without a predator present were investigated by playing back calls to 13 different groups (eight groups on the ranch and five groups in the park). However, not all the call types were played to all of the groups. Some call

types were tested in the same group more than once, but each time on a different subject (table 1). Only adult individuals were chosen as subjects. Because 11 call types were tested and each was tested in at least six groups, some individuals were used as subjects for more than one call type (however, the same subject was only used a maximum of three times in all of the experiments). Eighty-five subjects were tested in a total of 144 playbacks. At least six different examples of a call type were tested. High quality recordings of adult individuals in the same group were typically chosen as the playbacks were conducted. The calls were played from a Sony DAT recorder (Sony Corporation, Tokyo, Japan) connected to a Sony walkman SR A60 speaker, and responses were filmed with a Sony video high 8 camera or Panasonic digital video camera when possible (in eight out of the 144 experiments we were not able to film major parts of the response for technical reasons, and we used our notes made during the playbacks for the analysis). We adjusted the volume of the calls to the amplitude observed for calls given during naturally occurring predator encounters. All playbacks were conducted on suricates foraging at least 50 m from burrow systems and 10 m from boltholes within a range of 10–20 m of the loudspeaker. We only performed a playback if there had not been a predator encounter or group encounter during the previous 30 min. The subject was filmed for at least 30 s before the call was played and its response for a minimum of 30 s or until it relaxed. In order to minimize habituation to the playbacks, one playback experiment was typically performed per foraging session and the next was conducted 5–7 days later in the same group.

(iii) *Statistical analysis*

We analysed the response of the subject that had been filmed for each playback experiment. We then used a logistic regression model of Spss v. 10.0 in order to test the influence of predator type and the level of urgency on the frequency of specific responses. The duration of the time to relax was analysed by performing ANOVA after a logarithmic transformation of the data in order to fulfil the requirement of the data being normally distributed (Sokal & Rohlf 1995).

3. RESULTS

On the occasions when the suricates showed a response, they always did so immediately within the first few calls (2 s) of a playback. Although the only shelters for suricates are boltholes, it was possible to classify their responses within different categories. Suricates responded to the alarm calls by looking up briefly, rearing up on their hind legs to scan their surroundings, moving to a shelter and scanning the surrounding area, running back to a bolthole or burrow system without looking around or approaching the loudspeaker. Only one of these responses was possible for the analysis. The additional responses analysed, i.e. scanning the sky, gathering together, moving away together and erecting their tails and fur, did not exclude other responses. We included longer time-windows for these responses than just the first few seconds. We limited the response time for the response of scanning the sky to 20 s because they showed this behaviour either when rearing up or after they had run to a bolthole. We scored all the other non-exclusive responses if we observed them from any time when playing the call until the individual resumed foraging.

(a) *Responses to predator type-specific alarm calls*

The suricates responded in qualitatively different ways to the aerial, terrestrial and recruitment calls (tables 2 and 3). A few subjects showed no response to the aerial predator calls, but most of them either interrupted foraging and scanned the area or immediately ran to the next bolthole without first looking around. Individuals crouched down on the ground before they ran to a bolthole or resumed foraging again quite quickly in one-third of the playbacks of the high urgency aerial call. Although the subject scanned the sky within 20 s after having heard the call in only 11 out of 54 playbacks, this was still significantly more often than for any other call-type category. The time to relax after a playback was significantly shorter for aerial calls than for any other call type (ANOVA of predator type, d.f. $=1$, $F=20.49$ and $p<0.0001$) (figure 1).

The suricates always interrupted foraging when they heard any of the terrestrial calls but, rather than running to the next bolthole, they moved in the direction of the loudspeaker, frequently scanning the area. The suricates typically gathered together 5–10 m away from the loudspeaker and often marked each other before they then either moved away together to the next bigger burrow system in 15 out of 24 experiments or resumed foraging again quite quickly. The time to relax was much longer than after an aerial call (Bonferroni *post hoc* test of terrestrial versus aerial calls, $p<0.0001$) (figure 1).

A different response was shown to the recruitment calls. When they heard the calls, the suricates erected their tail and often also their hair and approached the loudspeaker slowly. In two cases, when there was a bolthole between their location and the loudspeaker, they would first go down to inspect the bolthole and then walk over to the loudspeaker. The suricates began to sniff around the area after approaching the loudspeaker and often marked each other before they resumed foraging. The time to relax was substantially longer than after aerial calls, but it was approximately the same as after hearing a terrestrial call (Bonferroni *post hoc* test of recruitment versus terrestrial calls, $p=0.37$ and Bonferroni *post hoc* test of recruitment versus aerial calls, $p=0.0008$) (figure 1).

(b) *Influence of response urgency of the alarm calls on the response*

The acoustic variation representing the level of urgency in the alarm calls influenced the responses of the suricates to the calls in a less obvious way than did the features that changed with predator type. Nevertheless, calls of low urgency of any predator category caused the subjects just to scan the area and not to move or run for shelter (tables 2 and 3). Furthermore, the time to relax increased substantially from the low to medium and again to the high level of urgency in the case of the terrestrial and recruitment calls (ANOVA of urgency d.f. $=1$, $F=18.24$ and $p<0.0001$) (figure 1). This was not the case for the aerial calls. This might be because the high-level urgency call elicited a rather different response from the subjects in that they crouched flat on the ground and, when they realized that nothing was happening, they resumed foraging again. On the other hand, running back to a bolthole in response to

Table 2. The number of subjects (n) and the percentages showing the different response categories to the playbacks of the different call types.

(The values marked by asterisks indicate the most common responses shown to the different call types. Column headings: 1, no response; 2, scanning the area; 3, crouching down; 4, scanning the sky; 5, moving to shelter and scanning area; 6, running for shelter without looking around; 7, gathering together; 8, moving away together; 9, approaching loudspeaker; 10, erecting tail; 11, erecting fur.)

				responses observed										
call type	predator type	level of urgency	total number of playbacks	1 n(%)	2 n(%)	3 n(%)	4 n(%)	5 n(%)	6 n(%)	7 n(%)	8 n(%)	9 n(%)	10 n(%)	11 n(%)
low urgent aerial	aerial	low	6	0	3(50)*	0	1(17)	1(17)	2(33)*	0	0	0	0	0
medium urgent aerial	aerial	medium	18	0	7(39)*	1(6)	3(22)*	1(6)	9(50)*	6	6	0	0	0
high urgent aerial	aerial	high	12	0	3(25)*	4(33)*	2(17)	2(17)	3(25)*	0	0	0	0	0
low urgent terrestrial	terrestrial	medium	6	0	2(33)*	0	0	4(67)*	0	3(50)*	2(33)*	0	0	0
medium urgent terrestrial	terrestrial	medium	12	0	2(17)	1(8)	0	9(75)*	1(8)	9(75)*	7(58)*	2(17)	0	0
high urgent terrestrial	terrrestrial	high	6	0	0	0	0	5(83)*	1(17)	3(50)*	2(33)*	0	0	0
low urgent recruitment	recruitment	low	12	0	0	0	0	1(8)	0	3(25)*	0	11(92)*	11(92)*	6(50)*
high urgent recruitment	recruitment	high	12	0	0	0	0	0	0	11(92)*	0	12(100)*	12(100)*	12(100)*
alert	aerial	low	18	4(22)	11(61)*	0	4(22)*	0	3(17)	0	0	0	0	0
moving animal	aerial/ terrestrial	low	18	5(33)	7(39)*	0	2(11)	5(33)*	1(6)	1(6)	0	0	0	0
panic	aerial/ terrestrial	high	12	1(7)	2(14)	0	0	0	9(75)*	0	0	0	0	0

Table 3. Statistics for the different response categories to the playbacks of the different call types.

(The statistics were performed with the original frequency data and show the results of the logistic regressions testing for differences in the responses for predator type and level of urgency. There were no data available for the no response category for any of the three variables. A dash indicates a p-value of < 0.0001. The values marked by asterisks indicate significant results. Column headings: 1, scanning the area; 2, crouching down; 3, scanning the sky; 4, moving to shelter and scanning area; 5, running for shelter without looking around; 6, gathering together; 7, moving away together; 8, approaching loudspeaker; 9, erecting tail; 10, erecting fur.)

	responses observed									
variable	1	2	3	4	5	6	7	8	9	10
predator type (d.f. = 2)										
χ^2-value	26.53	5.73	10.56	34.85	16.36	42.83	23.37	79.66	73.65	50.74
p-value	—*	0.057	0.005*	—*	—*	—*	—*	—*	—*	—*
urgency (d.f. = 2)										
χ^2-value	4.88	4.80	1.91	0.75	1.84	9.69	2.36	4.59	0.0	10.36
p-value	0.09	0.09	0.39	0.69	0.4	0.008*	0.31	0.10	1.0	0.006*
intercept (d.f. = 4)										
χ^2-value	18.25	10.71	14.63	35.38	22.13	45.69	29.01	92.00	100.51	70.65
p-value	—	0.03	0.006	—	—	—	—	—	—	—

low and medium urgency aerial calls required much more time.

The playback experiments of the three alarm-call types that were not elicited by specific predator types supported the pattern of responses seen in the predator-specific calls changing from the low to high level of urgency. The moving animal and alert calls, both of which are call types representing a low urgency situation, elicited the least strong responses in that the suricates more often either did not show any response at all or only scanned the area (tables 2 and 3). On the other hand, the panic call, which represents a high urgency situation,

evoked a very strong response. Only one subject did not interrupt its current occupation at all. Two subjects out of 12 only looked up briefly and then continued foraging, while nine out of 12 immediately ran to the next bolthole.

4. DISCUSSION

The suricates responded to the alarm-call playbacks with adaptive escape strategies that depended on two sorts of acoustic variation in call structure. Calls that were given in response to aerial predators, terrestrial predators, snakes and deposits evoked qualitatively different responses. Furthermore, within each of these call categories, calls that had been recorded in situations of high, medium and low urgency evoked the strongest, intermediate and the weakest responses, respectively.

These results indicate that call recipients are able to extract specific information about predator type and also the level of urgency from the acoustic structures of alarm calls in the absence of stimuli. Together with the high production specificity of aerial and terrestrial calls in response to different predator types, this suggests that suricates have evolved functionally referential alarm calls, as described for vervet monkeys (Seyfarth *et al*. 1980) and ring-tailed lemurs (Macedonia 1990). In addition, suricate alarm calls also vary depending on the level of response urgency, and receivers are able to extract that information and show adaptive responses within this dimension as well. However, the differences in the responses between low urgency and high urgency calls were less obvious than between the different predator types.

Although the receivers in this study showed a high perception specificity to the recruitment calls, it is not clear whether this call type is functionally referential or the expression of the affective state of a caller in order to manipulate other group members. Recruitment calls are not predator type specific as is the case with aerial or terrestrial calls (Manser 2001). Although they are mainly emitted in response to snakes and deposits, other animals such as suricates and terrestrial predators caught in boltholes elicit the same calls. Recruitment calls may denote the context of more or less stationary animals or deposits on the ground or in boltholes. It may be that it is not the predator type, but how a predator approaches or is encountered that elicits different call types (Evans 1997). However, the response of other group members to these calls for approaching the caller also supports the management hypothesis (Owings & Morton 1998). These calls may express the affective state of the caller who wants the group to gather together. More detailed observations and experiments need to be performed in order to draw conclusions on whether recruitment calls are the expression of the affective state of the caller or whether they denote a specific behaviour of an approaching animal.

A similar question arose in the analysis of the moving animal call. This call is given in response to different predator types and also non-dangerous animals, but only when they are moving (Manser 2001). The question that arose was whether the call was a higher urgency version of the alert call and the expression of the affective state of the caller or whether it was functionally referential and denoted a specific behaviour of the approaching animal. The responses to the playbacks of moving animal calls and alert calls were not obviously different, indicating a low perception specificity. Therefore, according to our analysis, this call type cannot be considered as functionally referential. The experiments suggest that the moving animal call is the expression of the affective state of the caller.

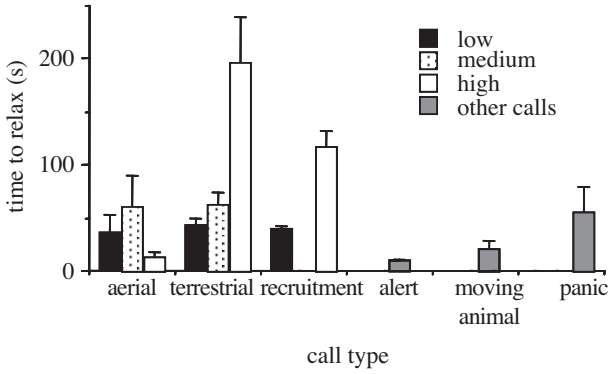

Figure 1. The time to relax (s) (mean ± s.e.) after the playbacks of the different call types.

(a) *The evolution of referential and response urgency alarm calls*

The reason why a species evolves functionally referential rather than only response urgency alarm calls has been explained by the different escape strategies that are needed (Macedonia & Evans 1993). While vervet monkeys respond by running up a tree in the case of the leopard call or running to the nearest bush when hearing an eagle alarm call (Seyfarth *et al*. 1980), the only way of escape in sciurid species is to run to a burrow. Suricates are in a similar situation to sciurids because their only escape from predators is retreating into a bolthole. However, they have evolved several different graded responses to intruders within this way of escape. On hearing an aerial alarm call they run to the nearest bolthole, since there is not much time to respond and the danger is usually only of short duration, whereas on hearing a terrestrial alarm call they gather together at the same safe place with the rest of the group in order possibly to leave the area together, since jackals in particular will watch a group for a long time (sometimes for longer than an hour) (M. B. Manser, personal observation). Similar subtle differences in ways of escape have been described in Belding's ground squirrels (*Spermophillus beldingi*), which run to the next burrow in the case of an aerial alarm and to burrow systems with more than one entrance in response to a terrestrial alarm (Sherman 1985). In addition, as described for Belding's ground squirrels, suricates not only emit calls in response to potential predators, but also to non-dangerous birds or herbivores approaching the group. The question therefore arises as to why suricates have evolved highly predator type-specific alarm calls, but Belding's ground squirrels have not.

Suricates are small terrestrial carnivores that dig for food in the sand. They travel from one sleeping burrow to the next and forage as a cohesive group up to several hundreds of metres away from larger burrow systems. However, they do maintain boltholes, which they

regularly dig out whenever they pass by. Unlike sciurids, suricates use a much larger territory and, if they encounter a terrestrial predator in part of the area, they often sneak away to another part several hundreds of metres away (M. B. Manser, unpublished data). By moving away from a stationary predator, either terrestrial or a perched raptor, they can resume their foraging in another area earlier than if they simply barked at the predator (Manser 1998) and waited for it to leave. However, this escape strategy requires coordination of their movements because, for each individual, losing the group could have severe consequences. Single individuals and small groups suffer a much higher predation rate than larger groups (Clutton-Brock *et al*. 1999). This might explain why, in the case of a terrestrial call, members first gather together, mark each other and then finally move to the next bigger burrow system together.

The advantage of emitting calls that are not only in response to potential predators but to any approaching non-dangerous animal becomes obvious when we consider the habitat that suricates occupy and their diet. They live in open semi-desert areas and dig for mobile prey, such as scorpions, small reptiles and insect larvae in the sand (Doolan & MacDonald 1996). Therefore, when they search for food they have their head on the ground or in digging holes and cannot see very far around them. By individuals emitting calls with specific information about predator type, the risk level of the animal and the level of urgency, they are able to adjust their responses. If suricates had to run for shelter each time an animal approached their foraging efficiency would drop substantially. The mobile prey that a suricate had just pursued would most probably have moved away by the time it returned to the spot after an alarm call. Therefore, in contrast to sciurids, suricates inhabiting large home ranges and digging for moving prey live under pressure to coordinate their movements and their vigilance behaviour in order to increase their foraging efficiency (Manser 1999).

The pressure for maintaining group cohesion and coordinating their vigilance behaviour might explain why suricates have evolved functionally referential alarm calls that also convey information about the level of urgency of the situation. The fitness benefit for suricates of using different escape strategies, although they are much more subtle than in vervet monkeys, may have been enough to evolve different call types with specific information. Subtle differences in their responses to different predator types may not be as important for some sciurid species, as the pressure to coordinate their group movements as a cohesive unit is not as high. Therefore, the evolution of functionally referential alarm calls in a species may not only depend on the presence of predators with different hunting strategies, but also on the social complexity under which the species is living (Blumstein & Armitage 1997*b*).

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BULLETIN OF THE TORREY BOTANICAL CLUB

VOL. 107, NO. 1, pp. 9–18 JANUARY–MARCH 1980

Leaf morphology in *Arenaria patula* and *Lonicera japonica* along a pollution gradient

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CAIAZZA, NICHOLAS A., JR., and JAMES A. QUINN. (Dept. Bot., Rutgers Univ., Piscataway, N.J. 08854). Leaf morphology in *Arenaria patula* and *Lonicera japonica* along a pollution gradient. Bull. Torrey Bot. Club 107: 9–18. 1980.—Certain plant species have persisted in denuded areas subjected to heavy metals (Zn, Cd, Pb, Cu) and SO_2 air pollution from two zinc smelters in Palmerton, Pennsylvania. The objectives of this research were to determine if correlations existed between the degree of environmental pollution and changes in leaf morphology along a local pollution gradient, and to determine the relative importance of genetic and environmental components responsible for the observed variations in leaf phenotypes. Leaves and epidermal peels from field samples of *Arenaria patula* and *Lonicera japonica* were examined microscopically. Sample sites were chosen to coincide with a previously documented air pollution gradient, and field conditions were monitored. Although stomatal size and leaf volume were not significantly different among populations of a species in the field, those populations of *Arenaria* and *Lonicera* exposed to the highest concentrations of pollutants exhibited the lowest stomatal density and the highest trichome density. Such alterations in leaf morphology should reduce the penetration of gaseous, and especially particulate matter, into the mesophyll and thus reduce susceptibility to pollution damage. Comparisons of results from the field with those of common environments (greenhouse and greenhouse courtyard) indicated phenotypic plasticity as the source of most of the observed field differences in *Arenaria* and *Lonicera;* however, they also provided some evidence for genetic dissimilarity in *Lonicera* populations in stomatal and trichome densities.

Key words: *Arenaria patula;* leaf morphology; *Lonicera japonica;* phenotypic plasticity; pollution gradient.

Adaptive differences in leaf morphology with respect to environmental factors have been reported in several plant species, either as population differentiation in diverse habitats (Briggs and Walters 1969), or as seasonal modifications within individuals (Regehr and Bazzaz 1976; Smith and Nobel 1977). Pollution as a stress has also received recent attention by Sharma and

along with increasing trichome length and density, in polluted (city) environments was the most common trend exhibited by populations of two clover species (*Trifolium pratense* [2] and *T. repens*) in Tennessee (Sharma and Butler 1973, 1975) and by sugar maple (*Acer saccharum*) in and around Montreal, Canada (Sharma 1975). In addition, populations of sweet-

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Adaptive differences in leaf morphology with respect to environmental factors have been reported in several plant species, either as population differentiation in diverse habitats (Briggs and Walters 1969), or as seasonal modifications within individuals (Regehr and Bazzaz 1976; Smith and Nobel 1977). Pollution as a stress has also received recent attention by Sharma and Tyree (1973), Sharma and Butler (1973, 1975), and Sharma (1975). These workers have correlated differences in leaf characteristics in plant populations with environmental changes along implied air pollution gradients. A decrease in stomatal density, along with increasing trichome length and density, in polluted (city) environments was the most common trend exhibited by populations of two clover species (*Trifolium pratense* [2] and *T. repens*) in Tennessee (Sharma and Butler 1973, 1975) and by sugar maple (*Acer saccharum*) in and around Montreal, Canada (Sharma 1975). In addition, populations of sweetgum (*Liquidambar styraciflua*) in Kentucky and Tennessee showed an increased trichome density in the urban sites (Sharma and Tyree 1973).

Sharma (1975) theorized that decreased stomatal density would limit gas exchange, thereby reducing exposure of moist, more susceptible inner leaf surfaces to the damaging effects of pollutants. Increased pubescence may act as a filter, screening out particulate matter and prohibiting it from entering stomata. Sharma and Butler (1973) also suggested that increased pubescence would reduce the amount of solar

[1] We are indebted to the Soil Testing Laboratory, Soils and Crops Department, Rutgers University, for assistance in soil analyses. We are also grateful for the helpful suggestions of Drs. Eileen Brennan, Marilyn Jordan, and Barbara Palser.

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[2] Nomenclature according to Gleason and Cronquist (1963).

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radiation incident upon the leaf surface. Such alteration of the leaf's energy budget might decrease leaf temperature, thus slowing down metabolism. This could be adaptive, considering Treshow's (1970) observation that air pollution damage is decreased in leaves with reduced metabolic rates.

Ideally, a population study concerning alterations in leaf morphology in association with a pollution gradient requires a thoroughly documented, long-persisting gradient. Such a study area exists at Palmerton, Pennsylvania, where zinc ore has been smelted since 1898 with the smelter effluents (SO_2, oxides and particulate matter of Zn, Cd, Pb, and Cu) producing pronounced vegetation damage. Extensive studies (T. H. Nash 1971, 1975; E. H. Nash 1972; Buchauer 1973; and Jordan 1975) have measured and plotted distinct air and soil pollution gradients emanating from the smelter area. The soil pollution (Zn, Cd, Pb, and Cu) is a result of and coincides with the air pollution gradient and can be thought of as a long-term indicator of air pollution levels.

Only a limited number of plant species occur in the polluted areas near the smelters. This paper reports studies on two of the most common, *Arenaria patula* and *Lonicera japonica*. Both species have demonstrated the general ability to colonize disturbed or polluted sites and might therefore exhibit adaptive populational differences in leaf morphology. *Arenaria,* a winter annual of the Caryophyllaceae, is very abundant in the denuded areas in and near Lehigh Gap even though its normal range does not include Pennsylvania (Buchauer 1971). The *Arenaria* at Palmerton has a high zinc tolerance, and its relative abundance may be due to a lack of competition from species that would normally invade such denuded areas (Buchauer 1971). Seedlings of *Arenaria* produce rosettes in the fall, and these rosettes then bolt the following spring to form short (usually under 30 cm in height) bushy plants that flower in early summer. *Lonicera japonica* (Caprifoliaceae), Japanese honeysuckle, is a trailing and climbing woody vine, native to eastern Asia, which has spread rapidly over a wide area of eastern North America since its introduction to the United States in 1806 (Slezak 1976). Japanese honeysuckle has been able to adjust or adapt to a wide variety of habitats and is easily established on poor soils and disturbed sites.

The primary objective of this study was to determine if there is a correlation between the degree of environmental pollution and certain features of adult leaf morphology within these two species along a documented pollution gradient. A second objective was to determine whether observed variability in leaf characters could be attributed to the phenotypic flexibility of individuals, or to genetic differences between them.

Materials and methods. THE STUDY AREA AND THE SAMPLE SITES. The town of Palmerton lies in a valley between Blue Mountain and Stony Ridge (Fig. 1). Blue Mountain (part of the Appalachian Range) consists mainly of Silurian conglomerate, with Martinsburg shale lying to the south and red siltstones and shales to the north. Most soils of the area were formed from colluvium and glacial till (Fisher *et al.* 1962). The mean yearly precipitation at Palmerton is 106.4 cm (climatological standard normal based on the period 1941 to 1970). Winds in the area are variable, but come predominantly from the northwest.

The New Jersey Zinc Company has smelted zinc in Palmerton since 1898 when the west plant was built on the north bank of the Lehigh River (Buchauer 1971). In 1911, the east plant was constructed south of Aquashicola Creek (Fig. 1). Zinc oxide fumes and particulate matter, as well as oxides of Cd, Pb, and Cu, are released from ore roasting processes at both plants. These oxides, along with SO_2 gas, are incompletely recovered by pollution control devices and released into the atmosphere (Jordan 1975). These smelters are the only significant point sources of air pollution within 30 km of Palmerton.

The lower north slope of Blue Mountain at east Lehigh Gap, against which prevailing winds (NW $\rightarrow$ SE) concentrate smelter effluents, shows the most extensive vegetation damage of the area (Buchauer 1971). Rotting stumps are all that is left of the original chestnut-oak forest, and the remaining woody vegetation consists almost exclusively of *Sassafras albidum* root sprouts. The herb layer is almost totally dominated by *Arenaria patula,* although some lichens and mosses remain

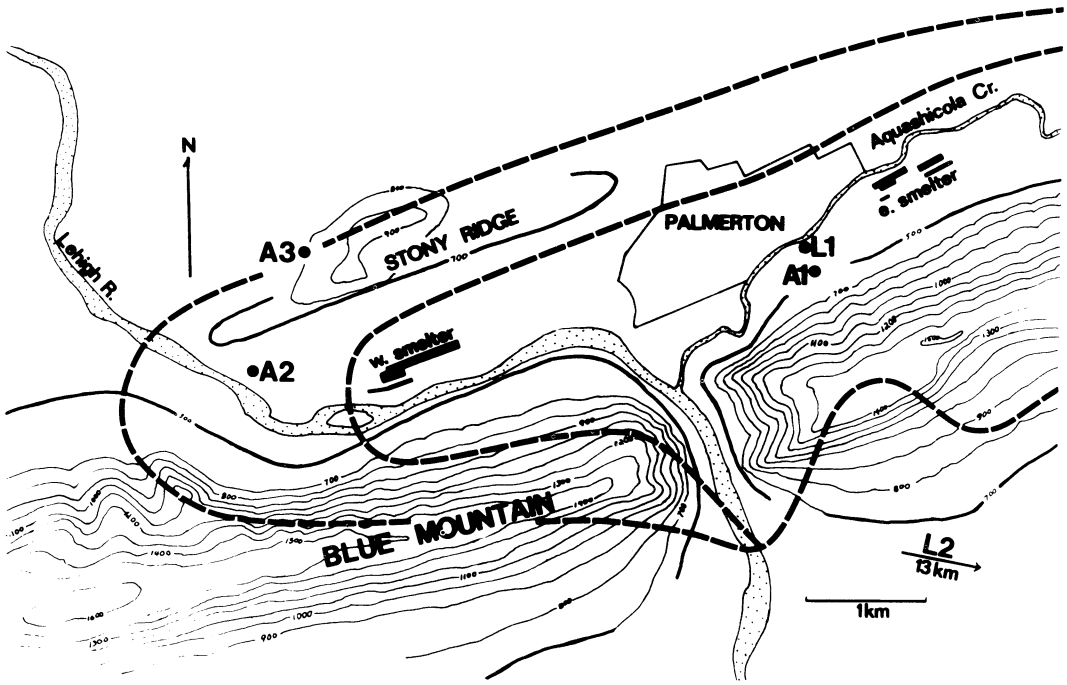

Fig. 1. The Lehigh Water Gap and vicinity. Included are Palmerton, the smelters, contour lines (el. in ft), and the *Arenaria* (A) and *Lonicera* (L) sample sites. The heavy dashed lines show the areas of high (center area including A1 and L1), intermediate (second ring including A2), and low (outside of dashed lines) sulfation values for 1970 (Nash, 1971). According to Nash (1975), the high area had sulfation rates exceeding 9.0 μg $SO_3/cm^2 \cdot$day for at least 2 mo, while in the low area "clean" air values were consistently recorded.

(Nash 1975). Buchauer (1971) estimated the Zn content in the soil at east Lehigh Gap to be as high as 11,000 kg/ha. Cd, Pb, and Cu are also present in elevated concentrations, but at much lower levels than Zn. As one moves away from Palmerton along the north slope or ridge of Blue Mountain, there is a smooth decrease in heavy metal concentrations (Buchauer 1973). SO_2 concentrations drop off more quickly and are above normal levels only in the highly polluted areas adjacent to the smelters (Fig. 1).

The sample sites were chosen to coincide with the above documented air and soil pollution gradients. *Arenaria* was studied at three field sites (A1, A2, A3), and *Lonicera* at two sites (L1, L2) (Fig. 1). The larger site numbers indicate increasing distances or protection from the pollution source. Site A1 is located on the north slope of Blue Mountain at east Lehigh Gap (elevation 490 ft or 149 m) in the region of greatest community damage. Site L1 is situated at the base of this slope (el. 400 ft or 122 m) on the south bank of Aquashicola Creek. Site A2 is 1 km west and upwind of the west smelter at el. 490 ft (149 m), while site A3 is 1 km northwest of this smelter at el. 800 ft (244 m), in a valley protected from smelter fumes by Stony Ridge. Site L2 is approximately 14 km southeast of Lehigh Gap in Beersville, Pennsylvania, just north of Route 248, on a slope at el. 490 ft (149 m).

Environmental data were collected from the field sites at different times during the 1977 and 1978 growing seasons. Soil samples, from 2 to 8 cm below the surface, were collected at all sites in the summer of 1977 for textural and chemical analyses. Textural analysis was by the hydrometer method (Bouyoucos 1953), while soil pH was determined in a 1:1 soil-water suspension using a Fisher's Acumet model 230 pH meter. Zinc and copper concentrations were determined in double acid extracts, using a Perkin-Elmer atomic absorption spectrophotometer. Soil moisture was determined for all sites on three separate

occasions by taking soil samples at 2 to 3 cm below the soil surface and oven-drying them at 104 C for 24 hr. Maximum and minimum temperatures at ground level over a 48-hr period were also taken at all sites on two occasions. Mercury (maximum) and alcohol (minimum) thermometers were placed on the ground and covered with leaf litter to prevent exposure to direct sunlight. Rate of evaporation at approximately 5 cm above ground level was measured with Piche evaporimeters (Livingston 1935) over a 48-hr period on two occasions.

FIELD MATERIALS. Plant cuttings from the three populations of *Arenaria* and the two of *Lonicera* were collected in June, 1977. All materials were taken from plants growing in as close to full sunlight as possible. *Arenaria* leaves were removed at or within one node of the point of branching of the main stem; *Lonicera* leaves were taken at least eight nodes back from the growing end of the vine.

With the exception of *Lonicera* trichomes, which were counted directly using a binocular dissecting microscope, stomatal and trichome densities and stomatal size were determined through the microscopic examination of leaf epidermal peels using Rhoplex, after the method of Horanic and Gardner (1967). Epidermal peels of entire *Arenaria* leaves were made, while peels from *Lonicera* leaves were made adjacent to the mid-vein of the leaf, midway along its length.

Stomatal densities were measured on all leaf surfaces on which stomata occur—upper and lower surfaces of *Arenaria* and the lower surface only of *Lonicera;* 8 plants/population, 2 leaves/plant, and 2 observations/leaf were utilized. Stomatal sizes (length of guard cells) were measured from one leaf surface in all field populations except A2.

Trichome densities were determined for both leaf surfaces of *Arenaria,* while only the upper epidermal hairs of *Lonicera* were counted since lower surface hairs occur discontinuously, being congregated on veins only. All trichome densities in June, 1977, were measured from 10 plants/population, 2 leaves/plant, and 2 observations/leaf.

Data were again collected from the field populations in July, 1978. Stomatal and trichome densities were determined as before, with the exception that all counts at this time were made from 6 newly selected plants/population, 2 leaves/plant, and 3 observations/leaf. In addition, data on leaf thickness and surface area for all populations were collected. Freehand cross-sections of *Arenaria* leaves were made at mid-length, and thickness of each section was determined microscopically using an ocular micrometer. *Lonicera* leaf thickness was measured in a similar manner, with cross-sections being cut adjacent to the mid-vein, midway along the length of the leaf. A Lambda Instrument Corporation electronic planimeter (model #LI-3000) was used to measure leaf area. All thickness and area measurements were taken from 12 plants/population and 2 leaves/plant.

GREENHOUSE AND COURTYARD OBSERVATIONS. *Lonicera* stem cuttings were taken from the field sites in June, 1977, and rooted in a 1:1 mixture of sand and potting soil in the greenhouse at the Nelson Biological Laboratories in Piscataway. In September, 1977, mature *Arenaria* fruiting stalks were collected from the field sites. Seeds and capsules were planted in flats with a 1:1 mixture of sand and potting soil in January, 1978. At this time, the *Lonicera* cuttings were trimmed back, and new foliage was allowed to grow for future sampling. Pots and flats were watered approximately every other day, maintaining a moderate soil moisture level (6 to 17% of dry soil weight). During the study period, the maximum daily temperature in the greenhouse ranged from about 26 to 31 C, while minimum daily temperature ranged from 18 to 22 C. The maximum during a 24-hr period generally exceeded the minimum by at least 8 degrees. Relative humidity ranged from 26 to 46%, and light intensity (measured on clear days between 11 a.m. and 1 p.m. with a Weston Illumination Meter #756) ranged from 4,400 to 5,000 ft-c. After 10 wk, density data were collected for *Arenaria* upper surface stomata and trichomes and for *Lonicera* upper surface trichomes and lower surface stomata. Sampling was similar to the field sampling in 1978. All stomata and trichome counts were taken from 6 plants/population, 2 leaves/plant, and 3 observations/leaf.

After data were taken in the greenhouse, all plants were moved outside to the greenhouse courtyard. *Arenaria* grew there

Table 1. Comparative data for five of the environmental factors monitored at the five study sites.

Sample site	Zinc (ppm)	Copper (ppm)	pH (1:1)	Mean soil moisture[1] (%)	Relative evaporation April 14–16 (ml)	Relative evaporation June 22–24 (ml)
A1	7,500	15	6.3	31.3	10.3	12.6
A2	3,344	10	6.2	22.3	6.7	9.1
A3	975	2.5	5.4	38.7	8.8	10.7
L1	5,875	3	6.8	22.0	—[2]	—[2]
L2	40	1	6.8	21.7	9.2	4.9

[1] Mean of three determinations at 2 to 3 cm below the soil surface.
[2] Data not available.

for approximately 12 wk before sampling, while *Lonicera* was sampled 16 wk after the transfer. Only leaves initiating and developing in the courtyard were used. The sampling and techniques for stomatal and trichome densities and for leaf thickness and area were those used on the July, 1978, field materials with the exception that *Arenaria* leaf surface area was not measured. The range of daily maximum temperature in the courtyard during most of the growth period was 18 to 37 C, while minimum temperatures ranged from 1 to 22 C. Relative humidity at mid-day ranged from 35 to 72%, and mid-day light intensities during clear weather ranged from 5,100 to 7,400 ft-c.

STATISTICAL ANALYSES. An analysis of variance model for hierarchal classifications (Snedecor and Cochran 1967) was utilized for all comparisons of three or more groups. When F was significant, differences between pairs of means were tested for significance using the LSD method. A *t*-test for groups of equal size (Snedecor and Cochran 1967) was utilized for all comparisons involving only two groups.

Results. ENVIRONMENT AT THE SAMPLE SITES. Sites A1, A2, and L1 occur in areas where Nash (1975) reported elevated SO_2 concentrations (Fig. 1). Zinc and copper concentrations in soil at the *Arenaria* and *Lonicera* sample sites were extremely high near the smelters, while dropping off in the distant sample areas (Table 1). These data correspond well with the heavy metal gradients determined by Buchauer (1973) for this area. A general increase in soil pH at *Arenaria* sites close to the smelters was recorded (Table 1), and this agrees with the findings of Buchauer (1971), who attributed the increase to the addition of large quantities of zinc oxide from smelter fumes to nearby soils. This amphoteric compound apparently acts as a base to neutralize the normally acidic soil. Presence of *Lonicera* at a site seemed to result in a higher pH, overriding possible location and texture effects, i.e., samples from similar areas adjacent to *Lonicera* sites had a lower pH.

The soil texture at all sites except L1 is loam, L1 being a loamy sand. The *Arenaria* sites showed differences in soil moisture for two of the three sampling days; site A2 consistently showed the driest soil, while A3 averaged the wettest (Table 1). The two *Lonicera* sites were quite similar in soil moisture.

Maximum and minimum temperature differences, measured twice over a 48-hr period, showed no consistent significant differences between sample sites. Data from Piche evaporimeters (Table 1) indicated that site A1 had the greatest evaporation of all sample sites, although it did not have the lowest soil moisture. This is consistent with the lack of a vegetational windbreak on the open slope at that site.

STOMATAL AND TRICHOME DENSITIES. Those *Arenaria* populations exposed to the lowest pollution levels had the highest stomatal densities in both the 1977 and 1978 field determinations (Table 2). The greater density of A3 was especially evident on the upper surface, where all population means were significantly different at the 0.05 level during 1977. In 1978 a similar range of difference occurred among the populations, although fewer differences were statistically significant because of in-

Table 2. Upper and lower leaf surface stomatal density for the three *Arenaria* populations growing under different conditions. Values are stomata/mm² surface area.

Upper surface				
	Field		Greenhouse	Courtyard
Population	1977	1978	March, '78	June, '78
A1	178.0^{a1,A2}	186.6^{a,B}	72.8^{a,C}	199.2^{a,D}
A2	199.9^{b,A}	211.5^{ab,A}	—[3]	—
A3	210.6^{c,A}	216.9^{b,B}	69.9^{a,C}	212.4^{a,AB}
Lower surface				
	Field		Greenhouse	Courtyard
Population	1977	1978	March, '78	June, '78
A1	129.6^{a1,A2}	135.8^{a,A}	—[3]	161.7^{a,B}
A2	136.1^{a,A}	158.1^{b,A}	—	—
A3	137.2^{a,A}	161.1^{b,B}	—	165.7^{a,B}

[1] Means in a vertical column followed by the same lower-case letter are not significantly different at the 0.05 level.
[2] Means in a horizontal row followed by the same upper-case letter are not significantly different at the 0.05 level.
[3] Data not available.

creased variance within populations. Trichome density showed the opposite trend—as pollution levels decreased, trichome densities decreased in all field comparisons (Table 3). All lower surface trichome density means during 1977 were significantly different, while only population A3, with its virtually glabrous upper leaf surfaces, was significantly different from other populations in upper surface means. The population values were more similar during 1978, while the upper surface densities were approximately double those of 1977.

Table 3. Upper and lower leaf surface trichome density for the three *Arenaria* populations growing under different conditions. Values are trichomes/mm² surface area.

Upper surface				
	Field		Greenhouse	Courtyard
Population	1977	1978	March, '78	June, '78
A1	0.38^{a1,A2}	0.67^{a,B}	0.68^{a,B}	0.59^{a,B}
A2	0.33^{a,A}	0.63^{a,A}	—[3]	—
A3	0.02^{b,A}	0.55^{a,B}	1.14^{a,C}	0.23^{b,D}
Lower surface				
	Field		Greenhouse	Courtyard
Population	1977	1978	March, '78	June, '78
A1	4.92^{a1,A2}	4.11^{a,B}	—[3]	0.08^{a,C}
A2	3.80^{b,A}	3.33^{a,A}	—	—
A3	2.90^{c,A}	3.14^{a,A}	—	0.20^{a,B}

[1] Means in a vertical column followed by the same lower-case letter are not significantly different at the 0.05 level.
[2] Means in a horizontal row followed by the same upper-case letter are not significantly different at the 0.05 level.
[3] Data not available.

Table 4. Lower surface stomatal and upper surface trichome density for leaves of two *Lonicera* populations growing under different conditions.

Lower surface stomata/mm²				
	Field		Greenhouse	Courtyard
Population	1977	1978	March, '78	July, '78
L1	$621.9^{a1,A2}$	$468.8^{a,B}$	$444.1^{a,C}$	$481.6^{a,D}$
L2	$840.5^{b,A}$	$499.8^{a,B}$	$578.2^{b,C}$	$668.6^{b,D}$
Upper surface trichomes/mm²				
	Field		Greenhouse	Courtyard
Population	1977	1978	March, '78	July, '78
L1	$2.55^{a1,A2}$	$0.98^{a,B}$	$2.43^{a,A}$	$1.78^{a,C}$
L2	$0.28^{b,A}$	$0.15^{b,B}$	$1.88^{a,C}$	$0.19^{b,B}$

[1] Means in a vertical column followed by the same lower-case letter are not significantly different at the 0.05 level.
[2] Means in a horizontal row followed by the same upper-case letter are not significantly different at the 0.05 level.

Stomatal density data from greenhouse and courtyard samplings of *Arenaria* (Table 2) showed some abrupt changes from field results. Upper surface stomatal density was much lower for leaves on plants grown in the greenhouse than for those sampled in the field, and when the greenhouse plants were moved outside into the courtyard for several weeks, upper stomatal densities changed again, either to a statistically significant new value (population A1), or back to one similar to that obtained in the field (population A3). Although greenhouse data were not taken, similar changes were evident in lower surface stomatal density. Trichome densities also showed some large changes from field to greenhouse or courtyard, especially for population A3 upper leaf and A1 lower leaf surfaces (Table 3).

The most polluted *Lonicera* site had lower stomatal density and greater pubescence in both 1977 and 1978 (Table 4). Cuttings from both field sites, when grown in the greenhouse or courtyard, showed means significantly different from those of the field plants, and the same individuals showed different values when moved from the greenhouse to the courtyard. Stomatal densities of plants from L1 and L2 in the greenhouse and courtyard remained significantly different, the L2 values always greater. Convergence of values was also lacking in the trichome greenhouse and courtyard data, although the greenhouse means were not significantly different at the 0.05 level.

STOMATAL SIZE. When stomatal size was measured on leaves from the 1977 field samplings, no significant difference was found between population means within a species. This indicates that the degree of leaf porosity when stomata were fully open probably did differ whenever stomatal density differed.

LEAF AREA, THICKNESS, AND VOLUME. The field populations of *Arenaria* showed a somewhat larger leaf area in the less polluted sites (means of A1 and A3 were significantly different); concurrently, leaves were slightly thinner (Table 5). These two opposing trends counteracted one another to provide volume means that did not differ significantly among sites. Courtyard means for leaf thickness differed significantly from the corresponding field values (Table 5), with leaves in the courtyard being 50% thicker. Within the courtyard, population means were equivalent.

No significant differences were detected between leaf area, thickness, or volume for leaves of *Lonicera* populations at the two field sites in 1978 (Table 6). While there were also no significant differences in the courtyard between populations in leaf area, thickness, or volume, there were significant differences between field and courtyard means for area and thickness for both pop-

Table 5. Mean leaf dimensions from *Arenaria* populations in the field (1978) and courtyard locations.

Location	Population	Leaf area (mm^2)	Leaf thickness (mm)	Leaf volume (mm^3)
Field	A1	20^{a1}	0.30^{a}	6.03^{a}
	A2	$21^{a,b}$	0.29^{a}	6.10^{a}
	A3	22^{b}	0.29^{a}	6.55^{a}
Courtyard	A1	—[2]	0.45^{b}	—
	A3	—	0.45^{b}	—

[1] Means in a vertical column followed by the same letter are not significantly different at the 0.05 level.
[2] Data not available.

ulations (Table 6). The changes were opposing, so that volume means were not significantly different from field values.

Discussion. RELATION OF LEAF MORPHOLOGY TO POLLUTANT LEVELS. Greater trichome density and lesser stomatal density in field populations of *Arenaria* and *Lonicera* were correlated with increased concentrations of environmental pollution. It is unlikely that this response was related to site differences in soil moisture, since the *Lonicera* sites showed similar moisture levels and since the trends in stomatal and trichome densities did not correspond to those of soil moisture or evaporation at the *Arenaria* sites (site A3 being wettest and A2 the driest). Stomatal and trichome densities usually increase or decrease simultaneously in response to moisture changes (Sharma and Dunn 1969; Bannister 1976; Ehleringer *et al.* 1976), whereas in our study they changed in opposite directions. In addition, the expectation of greater stomatal densities in open, sunny habitats of low humidity (Meidner and Mansfield 1968; Bannister 1976; Clay and Quinn 1978) was not realized in our polluted, open sites.

Stomatal size and leaf volume were not significantly different among populations of a species in the field. Differences in stomatal densities between the field populations were therefore neither a compensation for differences in size of individual stomata nor a compensatory response to the need for increased diffusion through a more massive leaf.

Although the effects of air and soil pollution were not separated in this study, selection for morphological characters has generally been found to be independent of tolerance to heavy metals in soils (Antonovics and Bradshaw 1970; Antonovics *et al.* 1971). The many differences at the same field site between 1977 and 1978 would also argue against a major effect of soil heavy metal concentrations on leaf morphological characters. The observed alterations in leaf morphology can thus be tentatively viewed as avoidance mechanisms for air pollution stress (Sharma 1975). Increased pubescence combined with decreased stomatal density should reduce the penetration of gaseous and especially particulate matter into the mesophyll of a leaf, a tissue which has been shown to be the most susceptible to several air pollutants (Solberg and Adams 1956; Treshow 1970). *Arenaria,* with its upper surface stomata, would probably benefit from morphological traits that limit penetration of heavy metal oxides and particulate matter and SO_2 into its leaves near the Palmerton smelters. Lower stomatal density in *Lonicera* may also be a response to air

Table 6. Mean leaf dimensions from *Lonicera* populations in the field (1978) and courtyard locations.

Population	Leaf area (mm^2)		Leaf thickness (mm)		Leaf volume (mm^3)	
	Field	Courtyard	Field	Courtyard	Field	Courtyard
L1	1232^{a1}	945^{b}	0.206^{a}	0.242^{b}	254.7^{a}	228.9^{a}
L2	1256^{a}	1014^{b}	0.197^{a}	0.235^{b}	249.2^{a}	238.2^{a}

[1] Means in a vertical column or horizontal row followed by the same letter are not significantly different at the 0.05 level.

pollution, but the increased trichome density measured at site L1 occurs on the upper leaf surface, away from the stomata, ruling out any filtering effect. The greater pubescence is more likely related to leaf energy budget considerations, e.g., greater insolation and the effects of higher leaf temperatures, but may also be significant in lowering metabolic rates in living cells and thus reducing their susceptibility to pollution damage (Sharma and Butler 1973).

VARIABILITY IN LEAF CHARACTERS—PHENOTYPIC FLEXIBILITY OR GENETIC DIFFERENCES? Significant differences within a population grown at different times (1977, 1978) or locations (field, greenhouse, courtyard) illustrate a high degree of phenotypic flexibility in leaf characters for both *Arenaria* and *Lonicera*. Stomatal and trichome densities, as well as leaf thickness and (for *Lonicera* only) leaf surface area, varied significantly within individuals in response to dissimilar environmental conditions. These results support the conclusions of Lewis (1972), who points out that, for several plant species, variations in leaf structure are common between different populations, and that these differences are often the result of the phenotypic plasticity of individuals.

Many of the differences between field populations were apparently environmentally-induced and disappeared, partially or completely, when plants from the field sites were grown under the common environments of the greenhouse and the courtyard. This was especially true for *Arenaria*. Seeds from sites A1 and A3 produced plants with similar (not significantly different) stomatal density in both the greenhouse and the courtyard. There was also some convergence and/or reversal of the field trends in the trichome densities. However, for *Lonicera* populations, comparisons of results from field and common environments provided some evidence for genetic dissimilarity. Stomatal densities of plants from the 2 sites remained significantly different in both the greenhouse and the courtyard. Convergence of trichome densities was also lacking. In this case, the individuals of each population may possess different genetically-fixed ranges of response, within which densities vary as the environment changes.

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Of nature trusts the Mind that builds for aye" —WORDSWORTH

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equipment, and to Dr. G. E. R. Deacon and the captain and officers of R.R.S. *Discovery II* for their part in making the observations.

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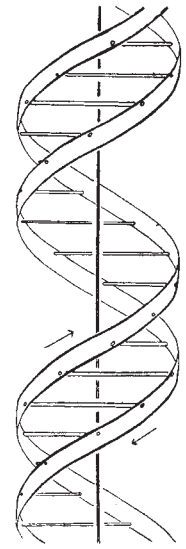
MOLECULAR STRUCTURE OF NUCLEIC ACIDS

A Structure for Deoxyribose Nucleic Acid

WE wish to suggest a structure for the salt of deoxyribose nucleic acid (D.N.A.). This structure has novel features which are of considerable biological interest.

A structure for nucleic acid has already been proposed by Pauling and Corey[1]. They kindly made their manuscript available to us in advance of publication. Their model consists of three intertwined chains, with the phosphates near the fibre axis, and the bases on the outside. In our opinion, this structure is unsatisfactory for two reasons: (1) We believe that the material which gives the X-ray diagrams is the salt, not the free acid. Without the acidic hydrogen atoms it is not clear what forces would hold the structure together, especially as the negatively charged phosphates near the axis will repel each other. (2) Some of the van der Waals distances appear to be too small.

Another three-chain structure has also been suggested by Fraser (in the press). In his model the phosphates are on the outside and the bases on the inside, linked together by hydrogen bonds. This structure as described is rather ill-defined, and for this reason we shall not comment on it.



This figure is purely diagrammatic. The two ribbons symbolize the two phosphate—sugar chains, and the horizontal rods the pairs of bases holding the chains together. The vertical line marks the fibre axis

We wish to put forward a radically different structure for the salt of deoxyribose nucleic acid. This structure has two helical chains each coiled round the same axis (see diagram). We have made the usual chemical assumptions, namely, that each chain consists of phosphate diester groups joining β-D-deoxyribofuranose residues with 3′,5′ linkages. The two chains (but not their bases) are related by a dyad perpendicular to the fibre axis. Both chains follow right-handed helices, but owing to the dyad the sequences of the atoms in the two chains run in opposite directions. Each chain loosely resembles Furberg's[2] model No. 1; that is, the bases are on the inside of the helix and the phosphates on the outside. The configuration of the sugar and the atoms near it is close to Furberg's 'standard configuration', the sugar being roughly perpendicular to the attached base. There is a residue on each chain every 3·4 A. in the z-direction. We have assumed an angle of 36° between adjacent residues in the same chain, so that the structure repeats after 10 residues on each chain, that is, after 34 A. The distance of a phosphorus atom from the fibre axis is 10 A. As the phosphates are on the outside, cations have easy access to them.

The structure is an open one, and its water content is rather high. At lower water contents we would expect the bases to tilt so that the structure could become more compact.

The novel feature of the structure is the manner in which the two chains are held together by the purine and pyrimidine bases. The planes of the bases are perpendicular to the fibre axis. They are joined together in pairs, a single base from one chain being hydrogen-bonded to a single base from the other chain, so that the two lie side by side with identical z-co-ordinates. One of the pair must be a purine and the other a pyrimidine for bonding to occur. The hydrogen bonds are made as follows: purine position 1 to pyrimidine position 1; purine position 6 to pyrimidine position 6.

If it is assumed that the bases only occur in the structure in the most plausible tautomeric forms (that is, with the keto rather than the enol configurations) it is found that only specific pairs of bases can bond together. These pairs are: adenine (purine) with thymine (pyrimidine), and guanine (purine) with cytosine (pyrimidine).

In other words, if an adenine forms one member of a pair, on either chain, then on these assumptions the other member must be thymine; similarly for guanine and cytosine. The sequence of bases on a single chain does not appear to be restricted in any way. However, if only specific pairs of bases can be formed, it follows that if the sequence of bases on one chain is given, then the sequence on the other chain is automatically determined.

It has been found experimentally[3,4] that the ratio of the amounts of adenine to thymine, and the ratio of guanine to cytosine, are always very close to unity for deoxyribose nucleic acid.

It is probably impossible to build this structure with a ribose sugar in place of the deoxyribose, as the extra oxygen atom would make too close a van der Waals contact.

The previously published X-ray data[5,6] on deoxyribose nucleic acid are insufficient for a rigorous test of our structure. So far as we can tell, it is roughly compatible with the experimental data, but it must be regarded as unproved until it has been checked against more exact results. Some of these are given in the following communications. We were not aware of the details of the results presented there when we devised our structure, which rests mainly though not entirely on published experimental data and stereochemical arguments.

It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.

Full details of the structure, including the conditions assumed in building it, together with a set of co-ordinates for the atoms, will be published elsewhere.

We are much indebted to Dr. Jerry Donohue for constant advice and criticism, especially on interatomic distances. We have also been stimulated by a knowledge of the general nature of the unpublished experimental results and ideas of Dr. M. H. F. Wilkins, Dr. R. E. Franklin and their co-workers at

King's College, London. One of us (J. D. W.) has been aided by a fellowship from the National Foundation for Infantile Paralysis.

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Molecular Structure of Deoxypentose Nucleic Acids

While the biological properties of deoxypentose nucleic acid suggest a molecular structure containing great complexity, X-ray diffraction studies described here (cf. Astbury[1]) show the basic molecular configuration has great simplicity. The purpose of this communication is to describe, in a preliminary way, some of the experimental evidence for the polynucleotide chain configuration being helical, and existing in this form when in the natural state. A fuller account of the work will be published shortly.

The structure of deoxypentose nucleic acid is the same in all species (although the nitrogen base ratios alter considerably) in nucleoprotein, extracted or in cells, and in purified nucleate. The same linear group of polynucleotide chains may pack together parallel in different ways to give crystalline[1–3], semi-crystalline or paracrystalline material. In all cases the X-ray diffraction photograph consists of two regions, one determined largely by the regular spacing of nucleotides along the chain, and the other by the longer spacings of the chain configuration. The sequence of different nitrogen bases along the chain is not made visible.

Oriented paracrystalline deoxypentose nucleic acid ('structure *B*' in the following communication by Franklin and Gosling) gives a fibre diagram as shown in Fig. 1 (cf. ref. 4). Astbury suggested that the strong 3·4-A. reflexion corresponded to the inter-nucleotide repeat along the fibre axis. The ~ 34 A. layer lines, however, are not due to a repeat of a polynucleotide composition, but to the chain configuration repeat, which causes strong diffraction as the nucleotide chains have higher density than the interstitial water. The absence of reflexions on or near the meridian immediately suggests a helical structure with axis parallel to fibre length.

Diffraction by Helices

It may be shown[5] (also Stokes, unpublished) that the intensity distribution in the diffraction pattern of a series of points equally spaced along a helix is given by the squares of Bessel functions. A uniform continuous helix gives a series of layer lines of spacing corresponding to the helix pitch, the intensity distribution along the nth layer line being proportional to the square of J_n, the nth order Bessel function. A straight line may be drawn approximately through

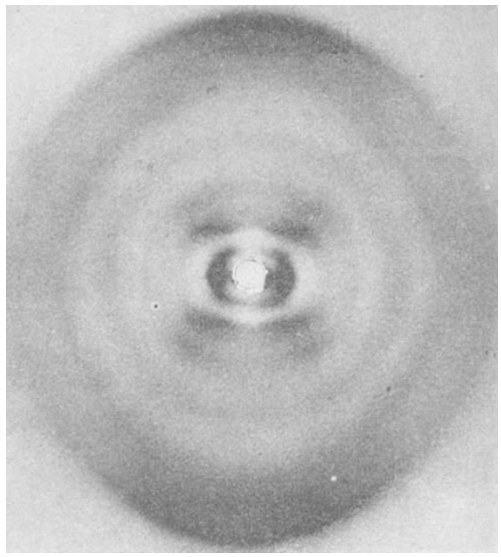

Fig. 1. Fibre diagram of deoxypentose nucleic acid from *B. coli*. Fibre axis vertical

the innermost maxima of each Bessel function and the origin. The angle this line makes with the equator is roughly equal to the angle between an element of the helix and the helix axis. If a unit repeats n times along the helix there will be a meridional reflexion (J_0^2) on the nth layer line. The helical configuration produces side-bands on this fundamental frequency, the effect[5] being to reproduce the intensity distribution about the origin around the new origin, on the nth layer line, corresponding to C in Fig. 2.

We will now briefly analyse in physical terms some of the effects of the shape and size of the repeat unit or nucleotide on the diffraction pattern. First, if the nucleotide consists of a unit having circular symmetry about an axis parallel to the helix axis, the whole diffraction pattern is modified by the form factor of the nucleotide. Second, if the nucleotide consists of a series of points on a radius at right-angles to the helix axis, the phases of radiation scattered by the helices of different diameter passing through each point are the same. Summation of the corresponding Bessel functions gives reinforcement for the inner-

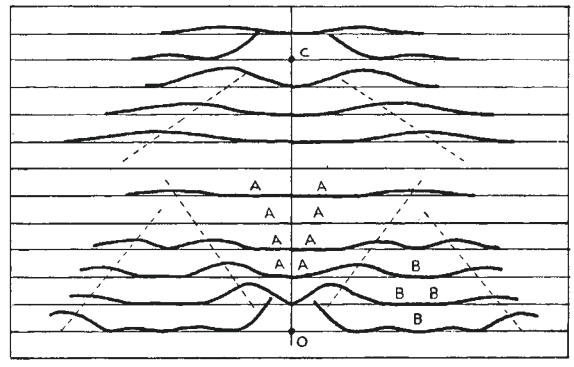

Fig. 2. Diffraction pattern of system of helices corresponding to structure of deoxypentose nucleic acid. The squares of Bessel functions are plotted about 0 on the equator and on the first, second, third and fifth layer lines for half of the nucleotide mass at 20 A. diameter and remainder distributed along a radius, the mass at a given radius being proportional to the radius. About C on the tenth layer line similar functions are plotted for an outer diameter of 12 A.

most maxima and, in general, owing to phase difference, cancellation of all other maxima. Such a system of helices (corresponding to a spiral staircase with the core removed) diffracts mainly over a limited angular range, behaving, in fact, like a periodic arrangement of flat plates inclined at a fixed angle to the axis. Third, if the nucleotide is extended as an arc of a circle in a plane at right-angles to the helix axis, and with centre at the axis, the intensity of the system of Bessel function layer-line streaks emanating from the origin is modified owing to the phase differences of radiation from the helices drawn through each point on the nucleotide. The form factor is that of the series of points in which the helices intersect a plane drawn through the helix axis. This part of the diffraction pattern is then repeated as a whole with origin at C (Fig. 2). Hence this aspect of nucleotide shape affects the central and peripheral regions of each layer line differently.

Interpretation of the X-Ray Photograph

It must first be decided whether the structure consists of essentially one helix giving an intensity distribution along the layer lines corresponding to $J_1, J_2, J_3 \ldots$, or two similar co-axial helices of twice the above size and relatively displaced along the axis a distance equal to half the pitch giving $J_2, J_4, J_6 \ldots$, or three helices, etc. Examination of the width of the layer-line streaks suggests the intensities correspond more closely to J_1^2, J_2^2, J_3^2 than to $J_2^2, J_4^2, J_6^2 \ldots$ Hence the dominant helix has a pitch of ~ 34 A., and, from the angle of the helix, its diameter is found to be ~ 20 A. The strong equatorial reflexion at ~ 17 A. suggests that the helices have a maximum diameter of ~ 20 A. and are hexagonally packed with little interpenetration. Apart from the width of the Bessel function streaks, the possibility of the helices having twice the above dimensions is also made unlikely by the absence of an equatorial reflexion at ~ 34 A. To obtain a reasonable number of nucleotides per unit volume in the fibre, two or three intertwined coaxial helices are required, there being ten nucleotides on one turn of each helix.

The absence of reflexions on or near the meridian (an empty region *AAA* on Fig. 2) is a direct consequence of the helical structure. On the photograph there is also a relatively empty region on and near the equator, corresponding to region *BBB* on Fig. 2. As discussed above, this absence of secondary Bessel function maxima can be produced by a radial distribution of the nucleotide shape. To make the layer-line streaks sufficiently narrow, it is necessary to place a large fraction of the nucleotide mass at ~ 20 A. diameter. In Fig. 2 the squares of Bessel functions are plotted for half the mass at 20 A. diameter, and the rest distributed along a radius, the mass at a given radius being proportional to the radius.

On the zero layer line there appears to be a marked J_{10}^2, and on the first, second and third layer lines, $J_9^2 + J_{11}^2$, $J_8^2 + J_{12}^2$, etc., respectively. This means that, in projection on a plane at right-angles to the fibre axis, the outer part of the nucleotide is relatively concentrated, giving rise to high-density regions spaced *c.* 6 A. apart around the circumference of a circle of 20 A. diameter. On the fifth layer line two J_5 functions overlap and produce a strong reflexion. On the sixth, seventh and eighth layer lines the maxima correspond to a helix of diameter ~ 12 A. Apparently it is only the central region of the helix structure which is well divided by the 3·4-A. spacing, the outer parts of the nucleotide overlapping to form a continuous helix. This suggests the presence of nitrogen bases arranged like a pile of pennies[1] in the central regions of the helical system.

There is a marked absence of reflexions on layer lines beyond the tenth. Disorientation in the specimen will cause more extension along the layer lines of the Bessel function streaks on the eleventh, twelfth and thirteenth layer lines than on the ninth, eighth and seventh. For this reason the reflexions on the higher-order layer lines will be less readily visible. The form factor of the nucleotide is also probably causing diminution of intensity in this region. Tilting of the nitrogen bases could have such an effect.

Reflexions on the equator are rather inadequate for determination of the radial distribution of density in the helical system. There are, however, indications that a high-density shell, as suggested above, occurs at diameter ~ 20 A.

The material is apparently not completely paracrystalline, as sharp spots appear in the central region of the second layer line, indicating a partial degree of order of the helical units relative to one another in the direction of the helix axis. Photographs similar to Fig. 1 have been obtained from sodium nucleate from calf and pig thymus, wheat germ, herring sperm, human tissue and T_2 bacteriophage. The most marked correspondence with Fig. 2 is shown by the exceptional photograph obtained by our colleagues, R. E. Franklin and R. G. Gosling, from calf thymus deoxypentose nucleate (see following communication).

It must be stressed that some of the above discussion is not without ambiguity, but in general there appears to be reasonable agreement between the experimental data and the kind of model described by Watson and Crick (see also preceding communication).

It is interesting to note that if there are ten phosphate groups arranged on each helix of diameter 20 A. and pitch 34 A., the phosphate ester backbone chain is in an almost fully extended state. Hence, when sodium nucleate fibres are stretched[3], the helix is evidently extended in length like a spiral spring in tension.

Structure *in vivo*

The biological significance of a two-chain nucleic acid unit has been noted (see preceding communication). The evidence that the helical structure discussed above does, in fact, exist in intact biological systems is briefly as follows :

Sperm heads. It may be shown that the intensity of the X-ray spectra from crystalline sperm heads is determined by the helical form-function in Fig. 2. Centrifuged trout semen give the same pattern as the dried and rehydrated or washed sperm heads used previously[6]. The sperm head fibre diagram is also given by extracted or synthetic[1] nucleoprotamine or extracted calf thymus nucleohistone.

Bacteriophage. Centrifuged wet pellets of T_2 phage photographed with X-rays while sealed in a cell with mica windows give a diffraction pattern containing the main features of paracrystalline sodium nucleate as distinct from that of crystalline nucleoprotein. This confirms current ideas of phage structure.

Transforming principle (in collaboration with H. Ephrussi-Taylor). Active deoxypentose nucleate allowed to dry at ~ 60 per cent humidity has the same crystalline structure as certain samples[3] of sodium thymonucleate.

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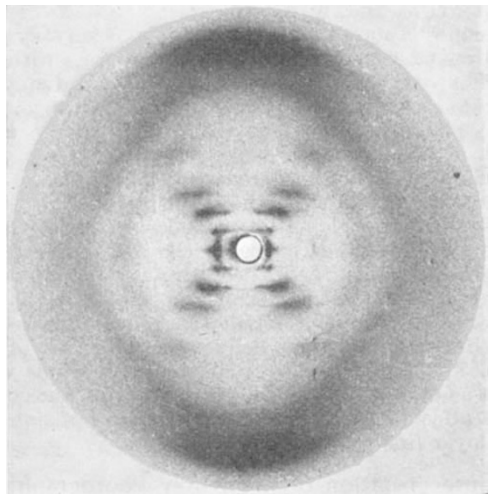
Molecular Configuration in Sodium Thymonucleate

Sodium thymonucleate fibres give two distinct types of X-ray diagram. The first corresponds to a crystalline form, structure *A*, obtained at about 75 per cent relative humidity; a study of this is described in detail elsewhere[1]. At higher humidities a different structure, structure *B*, showing a lower degree of order, appears and persists over a wide range of ambient humidity. The change from *A* to *B* is reversible. The water content of structure *B* fibres which undergo this reversible change may vary from 40–50 per cent to several hundred per cent of the dry weight. Moreover, some fibres never show structure *A*, and in these structure *B* can be obtained with an even lower water content.

The X-ray diagram of structure *B* (see photograph) shows in striking manner the features characteristic of helical structures, first worked out in this laboratory by Stokes (unpublished) and by Crick, Cochran and Vand[2]. Stokes and Wilkins were the first to propose such structures for nucleic acid as a result of direct studies of nucleic acid fibres, although a helical structure had been previously suggested by Furberg (thesis, London, 1949) on the basis of X-ray studies of nucleosides and nucleotides.

While the X-ray evidence cannot, at present, be taken as direct proof that the structure is helical, other considerations discussed below make the existence of a helical structure highly probable.

Structure *B* is derived from the crystalline structure *A* when the sodium thymonucleate fibres take up quantities of water in excess of about 40 per cent of their weight. The change is accompanied by an increase of about 30 per cent in the length of the fibre, and by a substantial re-arrangement of the molecule. It therefore seems reasonable to suppose that in structure *B* the structural units of sodium thymonucleate (molecules on groups of molecules) are relatively free from the influence of neighbouring



Sodium deoxyribose nucleate from calf thymus. Structure *B*

molecules, each unit being shielded by a sheath of water. Each unit is then free to take up its least-energy configuration independently of its neighbours and, in view of the nature of the long-chain molecules involved, it is highly likely that the general form will be helical[3]. If we adopt the hypothesis of a helical structure, it is immediately possible, from the X-ray diagram of structure *B*, to make certain deductions as to the nature and dimensions of the helix.

The innermost maxima on the first, second, third and fifth layer lines lie approximately on straight lines radiating from the origin. For a smooth single-strand helix the structure factor on the nth layer line is given by:

$$F_n = J_n(2\pi rR) \exp i\, n(\psi + \tfrac{1}{2}\pi),$$

where $J_n(u)$ is the nth-order Bessel function of u, r is the radius of the helix, and R and ψ are the radial and azimuthal co-ordinates in reciprocal space[2]; this expression leads to an approximately linear array of intensity maxima of the type observed, corresponding to the first maxima in the functions J_1, J_2, J_3, etc.

If, instead of a smooth helix, we consider a series of residues equally spaced along the helix, the transform in the general case treated by Crick, Cochran and Vand is more complicated. But if there is a whole number, m, of residues per turn, the form of the transform is as for a smooth helix with the addition, only, of the same pattern repeated with its origin at heights mc^*, $2mc^*$ . . . etc. (c is the fibre-axis period).

In the present case the fibre-axis period is 34 A. and the very strong reflexion at 3·4 A. lies on the tenth layer line. Moreover, lines of maxima radiating from the 3·4-A. reflexion as from the origin are visible on the fifth and lower layer lines, having a J_5 maximum coincident with that of the origin series on the fifth layer line. (The strong outer streaks which apparently radiate from the 3·4-A. maximum are not, however, so easily explained.) This suggests strongly that there are exactly 10 residues per turn of the helix. If this is so, then from a measurement of R_n the position of the first maximum on the nth layer line (for n 5≮), the radius of the helix, can be obtained. In the present instance, measurements of R_1, R_2, R_3 and R_5 all lead to values of r of about 10 A.

Since this linear array of maxima is one of the strongest features of the X-ray diagram, we must conclude that a crystallographically important part of the molecule lies on a helix of this diameter. This can only be the phosphate groups or phosphorus atoms.

If ten phosphorus atoms lie on one turn of a helix of radius 10 A., the distance between neighbouring phosphorus atoms in a molecule is 7·1 A. This corresponds to the P . . . P distance in a fully extended molecule, and therefore provides a further indication that the phosphates lie on the outside of the structural unit.

Thus, our conclusions differ from those of Pauling and Corey[4], who proposed for the nucleic acids a helical structure in which the phosphate groups form a dense core.

We must now consider briefly the equatorial reflexions. For a single helix the series of equatorial maxima should correspond to the maxima in $J_0(2\pi rR)$. The maxima on our photograph do not, however, fit this function for the value of r deduced above. There is a very strong reflexion at about 24 A. and then only a faint sharp reflexion at 9·0 A. and two diffuse bands around 5·5 A. and 4·0 A. This lack of agreement is, however, to be expected, for we know that the helix so far considered can only be the most important member of a series of coaxial helices of different radii ; the non-phosphate parts of the molecule will lie on inner co-axial helices, and it can be shown that, whereas these will not appreciably influence the innermost maxima on the layer lines, they may have the effect of destroying or shifting both the equatorial maxima and the outer maxima on other layer lines.

Thus, if the structure is helical, we find that the phosphate groups or phosphorus atoms lie on a helix of diameter about 20 A., and the sugar and base groups must accordingly be turned inwards towards the helical axis.

Considerations of density show, however, that a cylindrical repeat unit of height 34 A. and diameter 20 A. must contain many more than ten nucleotides.

Since structure B often exists in fibres with low water content, it seems that the density of the helical unit cannot differ greatly from that of dry sodium thymonucleate, 1·63 gm./cm.^{3} [1,5], the water in fibres of high water-content being situated outside the structural unit. On this basis we find that a cylinder of radius 10 A. and height 34 A. would contain thirty-two nucleotides. However, there might possibly be some slight inter-penetration of the cylindrical units in the dry state making their effective radius rather less. It is therefore difficult to decide, on the basis of density measurements alone, whether one repeating unit contains ten nucleotides on each of two or on each of three co-axial molecules. (If the effective radius were 8 A. the cylinder would contain twenty nucleotides.) Two other arguments, however, make it highly probable that there are only two co-axial molecules.

First, a study of the Patterson function of structure A, using superposition methods, has indicated[6] that there are only two chains passing through a primitive unit cell in this structure. Since the $A \rightleftharpoons B$ transformation is readily reversible, it seems very unlikely that the molecules would be grouped in threes in structure B. Secondly, from measurements on the X-ray diagram of structure B it can readily be shown that, whether the number of chains per unit is two or three, the chains are not equally spaced along the fibre axis. For example, three equally spaced chains would mean that the nth layer line depended on J_{3n}, and would lead to a helix of diameter about 60 A. This is many times larger than the primitive unit cell in structure A, and absurdly large in relation to the dimensions of nucleotides. Three unequally spaced chains, on the other hand, would be crystallographically non-equivalent, and this, again, seems unlikely. It therefore seems probable that there are only two co-axial molecules and that these are unequally spaced along the fibre axis.

Thus, while we do not attempt to offer a complete interpretation of the fibre-diagram of structure B, we may state the following conclusions. The structure is probably helical. The phosphate groups lie on the outside of the structural unit, on a helix of diameter about 20 A. The structural unit probably consists of two co-axial molecules which are not equally spaced along the fibre axis, their mutual displacement being such as to account for the variation of observed intensities of the innermost maxima on the layer lines ; if one molecule is displaced from the other by about three-eighths of the fibre-axis period, this would account for the absence of the fourth layer line maxima and the weakness of the sixth. Thus our general ideas are not inconsistent with the model proposed by Watson and Crick in the preceding communication.

The conclusion that the phosphate groups lie on the outside of the structural unit has been reached previously by quite other reasoning[1]. Two principal lines of argument were invoked. The first derives from the work of Gulland and his collaborators[7], who showed that even in aqueous solution the —CO and —NH_2 groups of the bases are inaccessible and cannot be titrated, whereas the phosphate groups are fully accessible. The second is based on our own observations[1] on the way in which the structural units in structures A and B are progressively separated by an excess of water, the process being a continuous one which leads to the formation first of a gel and ultimately to a solution. The hygroscopic part of the molecule may be presumed to lie in the phosphate groups ($(C_2H_5O)_2PO_2Na$ and $(C_3H_7O)_2PO_2Na$ are highly hygroscopic[8]), and the simplest explanation of the above process is that these groups lie on the outside of the structural units. Moreover, the ready availability of the phosphate groups for interaction with proteins can most easily be explained in this way.

We are grateful to Prof. J. T. Randall for his interest and to Drs. F. H. C. Crick, A. R. Stokes and M. H. F. Wilkins for discussion. One of us (R. E. F.) acknowledges the award of a Turner and Newall Fellowship.

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which has never been since surpassed. Dr. Schonland expressed disappointment that the membership in recent years has been but a little more than a thousand, for South Africa has expanded enormously since 1906 and with this expansion the need for, and potential value of, such a body as the Association. The general aims of the Association have not changed at all with the passing of years: "We exist," he said, "primarily to create and foster a scientific fraternity in South Africa, not to publish original work. We exist to provide a common meeting-ground for South African scientists and a forum for general discussion of the problems of this country from the scientific angle." He defended the use of Afrikaans by those who preferred it, for "we were intended by our founders to be parochial, and we should pride ourselves on being parochial. I would suggest that if we try to be anything else we will have mistaken our real aim".

Having thus firmly and, most people would agree, wisely placed the Association in its proper perspective, Dr. Schonland went on to make some concrete suggestions. The *South African Journal of Science* should have a series of semi-popular articles reviewing and surveying the new ideas of science and so bridge the gap between those who teach and do advanced research work and those who pay for it. This, he thought, is the proper function of the *Journal*, and it is but one aspect of the Association's duty, as representative of all sections of scientific opinion in South Africa, "to take a stronger, a more continuing and a more active interest in all scientific developments, national and university, in South Africa and to study carefully what is being done in other countries".

Besides his plea that the Association needs to form a standing committee to watch over scientific education in schools, Dr. Schonland suggested that the Association might consider taking a part in the formation of a body on the lines of the British Parliamentary and Scientific Committee and also help in the creation of better facilities for advanced research in South Africa. On this last-named point, he cited the instances of the National University in Canberra and the Institute for Advanced Studies in Dublin, but he made the interesting suggestion that a more acceptable solution might be the creation of a number of specialized institutes for advanced study, attached to and forming part of those universities which for one reason or another are best suited for them.

BASIS OF TECHNICAL EDUCATION

GENERAL education to-day should be planned so as to enable the ordinary citizen to adapt himself to the needs of technological society and to understand what is happening and what is required of him. This was the theme of an international conference convened by the United Nations Educational, Cultural and Scientific Organization at Unesco House in June 1950*.

Broadly, the Conference found that organized social foresight is essential to enable the educational system of a country to prepare children for the type of life and work they are likely to encounter, and that a substantial development of technical education is required at all levels: at present it is wholly inadequate for future needs, while the practical content of general education is also inadequate for the needs of future citizens of a technological society. The cultural content of technical education is also generally inadequate; technical education requires special consideration, and training for adaptability is an outstanding requirement in an age of ultra-rapid technological change. The education of women and girls also demands particular attention in view of their dual role as workers and home-makers, and improved administrative arrangements are essential if education is to fulfil its true function in such a society.

The report does not suggest that all these propositions apply equally to every country, though the Conference considered that, so far as its knowledge extended, they are generally valid for the world as a whole. The stress is laid on the need for adapting technology to man, not man to technology. The questions formulated in this report—and which merit attention in current discussions on the expansion of both technical and technological education in Great Britain—are raised in the belief that mastery of the machine by man is not an end in itself: it is a means to the development of man and of the whole society.

The distinction between technician and technologist is not always kept clear in this report, particularly in the chapter on the content of technical education. Nevertheless, the report directs attention to some fundamental issues which no sound policy for either type of education can disregard. In both fields it must be recognized that we are concerned not simply with the efficiency of production, but also with the fundamental attitude which the men and women of to-morrow will adopt in facing the problems of a technological society. Both, too, in seeking to foster flexibility, must recognize that flexibility is determined not only by education and training but also by social, economic and technical conditions; and the administrative measures required to ensure that education becomes more adapted to the needs of a changing technological society are themselves likely to be most effective when they are informal and varied rather than concentrated and uniform. The administrator, no less than the teacher and student, has need of frequent opportunities of contact with the industrial world, and requires experience of the difficulties and problems created by technological development in society; just as the teacher and student should keep abreast of developments in research and of practical applications in industry.

* Education in a Technological Society: a Preliminary International Survey of the Nature and Efficacy of Technical Education. (Tensions and Technology Series.) Pp. 76. (Paris: Unesco; London: H.M.S.O., 1952.) 200 francs; 4s.; 75 cents.

GENETICAL IMPLICATIONS OF THE STRUCTURE OF DEOXYRIBONUCLEIC ACID

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THE importance of deoxyribonucleic acid (DNA) within living cells is undisputed. It is found in all dividing cells, largely if not entirely in the nucleus, where it is an essential constituent of the chromosomes. Many lines of evidence indicate that it is the carrier of a part of (if not all) the genetic specificity of the chromosomes and thus of the gene itself.

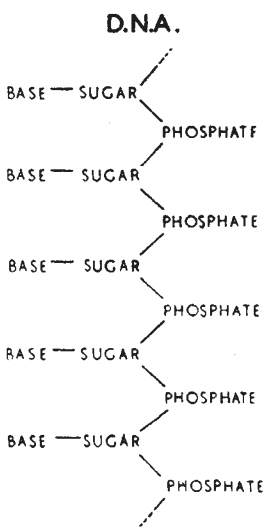

Fig. 1. Chemical formula of a single chain of deoxyribonucleic acid

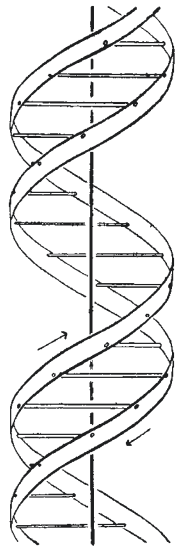

Fig. 2. This figure is purely diagrammatic. The two ribbons symbolize the two phosphate-sugar chains, and the horizontal rods the pairs of bases holding the chains together. The vertical line marks the fibre axis

Until now, however, no evidence has been presented to show how it might carry out the essential operation required of a genetic material, that of exact self-duplication.

We have recently proposed a structure[1] for the salt of deoxyribonucleic acid which, if correct, immediately suggests a mechanism for its self-duplication. X-ray evidence obtained by the workers at King's College, London[2], and presented at the same time, gives qualitative support to our structure and is incompatible with all previously proposed structures[3]. Though the structure will not be completely proved until a more extensive comparison has been made with the X-ray data, we now feel sufficient confidence in its general correctness to discuss its genetical implications. In doing so we are assuming that fibres of the salt of deoxyribonucleic acid are not artefacts arising in the method of preparation, since it has been shown by Wilkins and his co-workers that similar X-ray patterns are obtained from both the isolated fibres and certain intact biological materials such as sperm head and bacteriophage particles[2,4].

The chemical formula of deoxyribonucleic acid is now well established. The molecule is a very long chain, the backbone of which consists of a regular alternation of sugar and phosphate groups, as shown in Fig. 1. To each sugar is attached a nitrogenous base, which can be of four different types. (We have considered 5-methyl cytosine to be equivalent to cytosine, since either can fit equally well into our structure.) Two of the possible bases—adenine and guanine—are purines, and the other two—thymine and cytosine—are pyrimidines. So far as is known, the sequence of bases along the chain is irregular. The monomer unit, consisting of phosphate, sugar and base, is known as a nucleotide.

The first feature of our structure which is of biological interest is that it consists not of one chain, but of two. These two chains are both coiled around a common fibre axis, as is shown diagrammatically in Fig. 2. It has often been assumed that since there was only one chain in the chemical formula there would only be one in the structural unit. However, the density, taken with the X-ray evidence[2], suggests very strongly that there are two.

The other biologically important feature is the manner in which the two chains are held together. This is done by hydrogen bonds between the bases, as shown schematically in Fig. 3. The bases are joined together in pairs, a single base from one chain being hydrogen-bonded to a single base from the other. The important point is that only certain pairs of bases will fit into the structure. One member of a pair must be a purine and the other a pyrimidine in order to bridge between the two chains. If a pair consisted of two purines, for example, there would not be room for it.

We believe that the bases will be present almost entirely in their most probable tautomeric forms. If this is true, the conditions for forming hydrogen bonds are more restrictive, and the only pairs of bases possible are :

adenine with thymine ;
guanine with cytosine.

The way in which these are joined together is shown in Figs. 4 and 5. A given pair can be either way round. Adenine, for example, can occur on either chain ; but when it does, its partner on the other chain must always be thymine.

This pairing is strongly supported by the recent analytical results[5], which show that for all sources of deoxyribonucleic acid examined the amount of adenine is close to the amount of thymine, and the amount of guanine close to the amount of cytosine, although the cross-ratio (the ratio of adenine to guanine) can vary from one source to another. Indeed, if the sequence of bases on one chain is irregular, it is difficult to explain these analytical results except by the sort of pairing we have suggested.

The phosphate-sugar backbone of our model is completely regular, but any sequence of the pairs of bases can fit into the structure. It follows that in a long molecule many different permutations are possible, and it therefore seems likely that the precise sequence of the bases is the code which carries the genetical information. If the actual order of the

Fig. 3. Chemical formula of a pair of deoxyribonucleic acid chains. The hydrogen bonding is symbolized by dotted lines

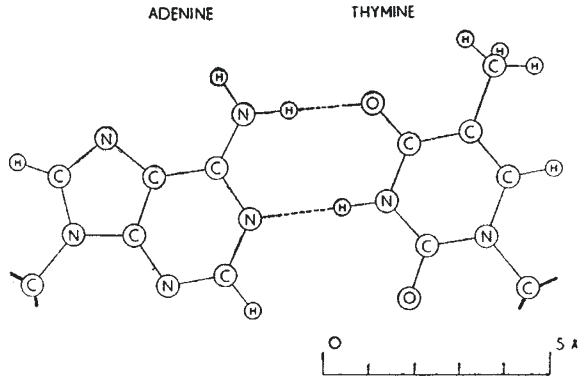

Fig. 4. Pairing of adenine and thymine. Hydrogen bonds are shown dotted. One carbon atom of each sugar is shown

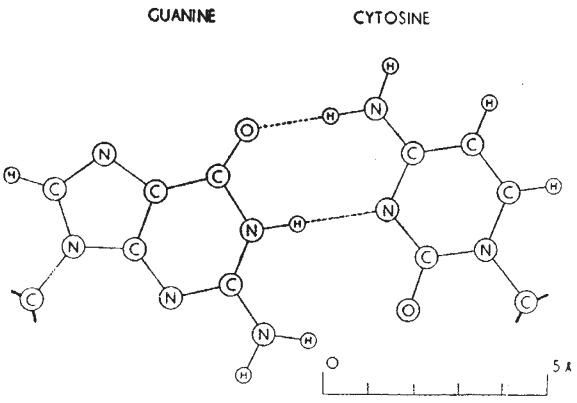

Fig. 5. Pairing of guanine and cytosine. Hydrogen bonds are shown dotted. One carbon atom of each sugar is shown

bases on one of the pair of chains were given, one could write down the exact order of the bases on the other one, because of the specific pairing. Thus one chain is, as it were, the complement of the other, and it is this feature which suggests how the deoxyribonucleic acid molecule might duplicate itself.

Previous discussions of self-duplication have usually involved the concept of a template, or mould. Either the template was supposed to copy itself directly or it was to produce a 'negative', which in its turn was to act as a template and produce the original 'positive' once again. In no case has it been explained in detail how it would do this in terms of atoms and molecules.

Now our model for deoxyribonucleic acid is, in effect, a *pair* of templates, each of which is complementary to the other. We imagine that prior to duplication the hydrogen bonds are broken, and the two chains unwind and separate. Each chain then acts as a template for the formation on to itself of a new companion chain, so that eventually we shall have *two* pairs of chains, where we only had one before. Moreover, the sequence of the pairs of bases will have been duplicated exactly.

A study of our model suggests that this duplication could be done most simply if the single chain (or the relevant portion of it) takes up the helical configuration. We imagine that at this stage in the life of the cell, free nucleotides, strictly polynucleotide precursors, are available in quantity. From time to time the base of a free nucleotide will join up by hydrogen bonds to one of the bases on the chain already formed. We now postulate that the polymerization of these monomers to form a new chain is only possible if the resulting chain can form the proposed structure. This is plausible, because steric reasons would not allow nucleotides 'crystallized' on to the first chain to approach one another in such a way that they could be joined together into a new chain, unless they were those nucleotides which were necessary to form our structure. Whether a special enzyme is required to carry out the polymerization, or whether the single helical chain already formed acts effectively as an enzyme, remains to be seen.

Since the two chains in our model are intertwined, it is essential for them to untwist if they are to separate. As they make one complete turn around each other in 34 A., there will be about 150 turns per million molecular weight, so that whatever the precise structure of the chromosome a considerable amount of uncoiling would be necessary. It is well known from microscopic observation that much coiling and uncoiling occurs during mitosis, and though this is on a much larger scale it probably reflects similar processes on a molecular level. Although it is difficult at the moment to see how these processes occur without everything getting tangled, we do not feel that this objection will be insuperable.

Our structure, as described[1], is an open one. There is room between the pair of polynucleotide chains (see Fig. 2) for a polypeptide chain to wind around the same helical axis. It may be significant that the distance between adjacent phosphorus atoms, 7·1 A., is close to the repeat of a fully extended polypeptide chain. We think it probable that in the sperm head, and in artificial nucleoproteins, the polypeptide chain occupies this position. The relative weakness of the second layer-line in the published X-ray pictures[3a,4] is crudely compatible with such an idea. The function of the protein might well be to control the coiling and uncoiling, to assist in holding a single polynucleotide chain in a helical configuration, or some other non-specific function.

Our model suggests possible explanations for a number of other phenomena. For example, spontaneous mutation may be due to a base occasionally occurring in one of its less likely tautomeric forms. Again, the pairing between homologous chromosomes at meiosis may depend on pairing between specific bases. We shall discuss these ideas in detail elsewhere.

For the moment, the general scheme we have proposed for the reproduction of deoxyribonucleic acid must be regarded as speculative. Even if it is correct, it is clear from what we have said that much remains to be discovered before the picture of genetic duplication can be described in detail. What are the polynucleotide precursors ? What makes the pair of chains unwind and separate ? What is the precise role of the protein ? Is the chromosome one long pair of deoxyribonucleic acid chains, or does it consist of patches of the acid joined together by protein ?

Despite these uncertainties we feel that our proposed structure for deoxyribonucleic acid may help to solve one of the fundamental biological problems—the molecular basis of the template needed for genetic replication. The hypothesis we are suggesting is that the template is the pattern of bases formed by one chain of the deoxyribonucleic acid and that the gene contains a complementary pair of such templates.

One of us (J.D.W.) has been aided by a fellowship from the National Foundation for Infantile Paralysis (U.S.A.).

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GEOPHYSICAL AND METEOROLOGICAL CHANGES IN THE PERIOD JANUARY–APRIL 1949

In a recent article[1] Lewis and McIntosh have considered the geophysical data for the period January–April 1949, which we presented in an earlier communication[2]. On the basis of certain probability criteria they appear to show that the apparent regular variations in ionospheric and meteorological phenomena which occurred in that period were not significant. We have studied their article and made a separate statistical analysis of the *unsmoothed* data, and conclude that in all respects our original suggestions seem to be valid.

In our original article we presented graphs showing five-day moving averages in four parameters: (*a*) ground pressure, p; (*b*) E-layer critical frequency, fE; (*c*) F-layer critical frequency, $fF2$; and (*d*) K-index of geomagnetic activity. The connexion between ionospheric and geomagnetic phenomena is well known. Thus, Appleton and Ingram[3] in 1935 established the correlation between geomagnetic activity and depressions in $fF2$. It is worthy of note that in the period under discussion the inverse correlation between K and $\Delta fF2$ is, as Lewis and McIntosh point out, considerably less striking than that between p and ΔfE (cf. Figs. 1 and 2 in our original article). It would seem, then, that if statistical analysis can be successfully applied to show that there is no significance between the variations in p and ΔfE, it is, *a fortiori*, evident that a similar analysis might, in the present instance, be used for discrediting the established relationship between K and $\Delta fF2$. Conversely, of course, the fact that a phenomenon appears to be statistically significant over a short period must likewise be treated with reserve. The need for the utmost care in the application and interpretation of statistical analyses to such a limited time series is thus clear.

From inspection of our graphs it seemed to us that, so far as p and ΔfE were concerned, the period was unusual in three respects: (i) there appeared to be four oscillations in ground pressure showing a progressive diminution of amplitude, with an average period of about 27 days; (ii) in like manner there appeared to be four marked oscillations of period about 27 days in ΔfE; (iii) oscillations (i) and (ii) appeared to be almost exactly out of phase. In addition, we noted that the period was characterized by an unusual 27-day recurrence of great sudden commencement (S.C.) magnetic storms.

In our original communication we merely directed attention to these matters, and suggested that there might be some connexion between them. We did not then suggest, nor do we now suggest, that from a period of length only four months any conclusions can be drawn regarding the general behaviour over a long period of any of the geophysical parameters considered. The severely limited number of observations available, together with the fact that there is considerable uncertainty about the correct statistical approach to time series analysis, seemed to us sufficient reason for not entering into an extended statistical analysis.

However, the contrary conclusions reached by Lewis and McIntosh (see below) have prompted us to re-examine the data. Briefly, their conclusions are: (i) the 27-day oscillation in ground pressure is of no significance, since the amplitude is no more than would be expected from mere chance considerations; (ii) the 27-day oscillation in ΔfE is probably significant; (iii) oscillations (i) and (ii) are exactly in anti-phase; (iv) there is no significant correlation coefficient between the p and ΔfE data; (v) our conclusions arise from smoothing of the data.

We shall now outline our own analysis. In various communications[4–6], Kendall has made it abundantly clear that most of the methods generally used for studying periodicities in time series (for example, periodograms, Fourier analysis, etc.) may yield very misleading results when applied to the kind of time series with which we are here concerned. He has also questioned the reliability of the usual significance tests for periodicities when applied in time series analysis. Kendall has shown that the most reliable approach is that of serial correlation coefficients as exhibited in the correlogram. He points out that although the correlogram may be insensitive, it does give a lower limit to the oscillatory effects, and that if it oscillates there is almost certainly some systematic oscillation in the primary series explored. Figs. 1 and 2 show the correlograms for Δp and ΔfE respectively for the period under consideration. In both of these the original *unsmoothed* data have been used.

It is important to note that there is a marked trend in the pressure data, and to eliminate this we have dealt with values of pressure departures, Δp (as with the fE data), rather than with the absolute magnitudes p. The oscillations in both correlograms are clear, with a maximum at 26–27 days in each case. These correlograms provide strong support for our original deductions (based, as they were, on simple inspection of graphs), and make it essential for us to repeat Lewis and McIntosh's calculations.

At the outset we must again stress that the pressure data exhibit a marked downward trend (approximately linear), and it is imperative initially to eliminate this before proceeding with any numerical analysis. It appears that Lewis and McIntosh have overlooked this point, and as a result have arrived at quite contrary conclusions. This will be clear from an examination of Table 1, in which we present the results of calculations made by us using (i) pressure, p, (ii) pressure departures, Δp, and (iii) fE departures, ΔfE. The nomenclature employed (c, φ, σ, etc.) is that used by Lewis and McIntosh.

Without going into details, it can be stated that there is little significant difference between the present results *using pressure*, p, and those given by Lewis and McIntosh. The slight differences in the values of amplitude c and first serial correlation coefficient r_1 are of no significance and can be ascribed to different ways of deducing the amplitude and phase

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5-Azacytidine stimulates fetal hemoglobin synthesis in anemic baboons

(globin genes/hypomethylation/cytidine analogue/gene expression)

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Communicated by Leon D. Jacobson, April 21, 1982

ABSTRACT **In an attempt to stimulate Hb F synthesis in baboons by means other than erythropoietic stress, we considered the possibility that an agent that inhibits methylation of CpG sequences in DNA may be effective. 5-Azacytidine, a cytosine analogue that cannot be methylated, is such an agent. Animals whose packed red cell volume was maintained at approximately 20% by bleeding were given 10 daily intravenous injections of the drug (6 mg/kg) in 12 days. Hb F levels in these animals started to increase on day 5 of this regimen and peak levels, which were 6–30 times higher than those produced by bleeding alone, occurred 5–7 days after the last dose of the drug. In animals previously identified as genetically "high" or "low" Hb F responders, the maximal Hb F levels were 70–85% and 35–40% respectively. In dose–response studies 5-azacytidine given daily at 3–4 mg/kg produced maximal Hb F increases. The drug did not increase the percentage (number) of Hb F-containing cells (F cells) beyond the maximal number achieved by bleeding alone and thus its main effect was to increase Hb F per F cell. The finding that Hb F synthesis can be modulated to such a high degree by a drug may have therapeutic implications—e.g., in sickle cell anemia, in which stimulation of Hb F synthesis may prevent sickling.**

The degree of methylation of CpG dinucleotide sequences of DNA has been shown to be important in the control of gene

in Hb F synthesis (7–9). The magnitude of this response (high or low) has been shown to be genetically determined (10, 11) and it appeared to be of interest to determine whether these genetic differences could be influenced by 5-azaC. Other myelosuppressive agents [hydroxyurea and 1-β-D-arabinofuranosylcytosine (araC, cytosine arabinoside)] were used in four baboons to test their effect on Hb F synthesis.

METHODS

Initially, four baboons (2, 3, 3, and 5 years old; weight 4–12 kg) were bled to reduce the packed erythrocyte volume (PCV) to 20% within 5 days. Two of the baboons had been found to be high Hb F responders and two were low responders (10, 11). The PCV of 20% was maintained for another 10 days by bleeding; during this time, Hb F levels were measured every day by alkali denaturation (12) to determine the extent of Hb F increase in each animal due to bleeding alone. There was an adequate reticulocyte response which plateaued at approximately 20%. 5-azaC (6 mg/kg) was injected intravenously on days 15–19 and 22–26. The PCV was maintained at 20% by daily removal of 10–20% of the blood volume. Blood cell counts (including platelets) were obtained daily with a Coulter Counter (model S plus). F cell number (percentage of erythrocytes containing reciprocal

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The degree of methylation of CpG dinucleotide sequences of DNA has been shown to be important in the control of gene activity, hypomethylation being associated with expressed genes (1–4). At this time it is not known which CpG sequences at specific positions in the genome are crucial to gene expression. The non-α-globin gene cluster has been recognized as being a suitable model for the investigation of this problem because it might be expected that the well-known changes in gene expression during ontogeny ($\gamma \rightarrow \delta\beta$) are related to reciprocal changes in methylation. However, both γ and β genes are hypomethylated in human fetal erythropoietic tissue (5). It therefore is likely that hypomethylation is not the only factor responsible for the changes in the expression of the globin genes during ontogeny. On the other hand, the fact that in adult erythropoietic tissue the CpG sequences associated with the γ regions are methylated suggests that methylation of DNA is a factor involved in the cessation of γ chain synthesis (<1%) in the adult (5).

It also offers an experimental design to test for the possibility that incorporation of 5-azacytidine (5-azaC), an analogue of cytosine that cannot be methylated, into DNA leads to γ-globin gene expression in the adult. We have chosen to use this agent *in vivo* in the baboon because this animal is a suitable model for the study of normal hemoglobin switching (6) and also because the reverse switch in the adult baboon has been shown to respond to erythropoietic stress with an increase in the number of Hb F-containing erythrocytes (F cells) and an increase in Hb F synthesis (7–9). The magnitude of this response (high or low) has been shown to be genetically determined (10, 11) and it appeared to be of interest to determine whether these genetic differences could be influenced by 5-azaC. Other myelosuppressive agents [hydroxyurea and 1-β-D-arabinofuranosylcytosine (araC, cytosine arabinoside)] were used in four baboons to test their effect on Hb F synthesis.

METHODS

Initially, four baboons (2, 3, 3, and 5 years old; weight 4–12 kg) were bled to reduce the packed erythrocyte volume (PCV) to 20% within 5 days. Two of the baboons had been found to be high Hb F responders and two were low responders (10, 11). The PCV of 20% was maintained for another 10 days by bleeding; during this time, Hb F levels were measured every day by alkali denaturation (12) to determine the extent of Hb F increase in each animal due to bleeding alone. There was an adequate reticulocyte response which plateaued at approximately 20%. 5-azaC (6 mg/kg) was injected intravenously on days 15–19 and 22–26. The PCV was maintained at 20% by daily removal of 10–20% of the blood volume. Blood cell counts (including platelets) were obtained daily with a Coulter Counter (model S plus). F cell number (percentage of erythrocytes containing reciprocal concentrations of Hb F and Hb A) was determined by the acid elution test (13). Globin chain synthesis was measured periodically in reticulocytes throughout the experimental period (6). One additional animal, which was not bled, received 5-azaC at 6 mg/kg each weekday for 2 weeks (10 injections) to determine whether Hb F increased in nonanemic animals. Hb F levels and synthesis were monitored as above.

After it had been established that 5-azaC at 6 mg/kg stimulated Hb F synthesis, a dose–response relationship was determined as follows. Two high Hb F responders and two low responders were treated with 5-azaC in the same way as the original four animals, except that 1 mg/kg was given daily for 5 days followed by 10 days of bleeding to maintain a PCV of 20%. This schedule then was repeated for dosages of 2, 3, and 4 mg/kg.

One animal was treated with hydroxyurea (25–50 mg/kg each weekday for 2 weeks) to determine whether myelosuppression alone (and achieved by a different molecular mechanism) might be responsible for increased Hb F synthesis. Two animals received hydroxyurea (25–50 mg/kg/day) followed 30 min later by 5-azaC (3 mg/kg) each weekday for 2 weeks. This regimen was used to determine the influence of hydroxyurea (which interferes with DNA synthesis by inhibiting ribonucleoside diphosphate reductase) on the effectiveness of 5-azaC in stimulating Hb F production. Another animal received araC

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Abbreviations: 5-azaC, 5-azacytidine; F cells, erythrocytes containing Hb F; PCV, packed erythrocyte volume; araC, 1-β-D-arabinofuranosylcytosine.

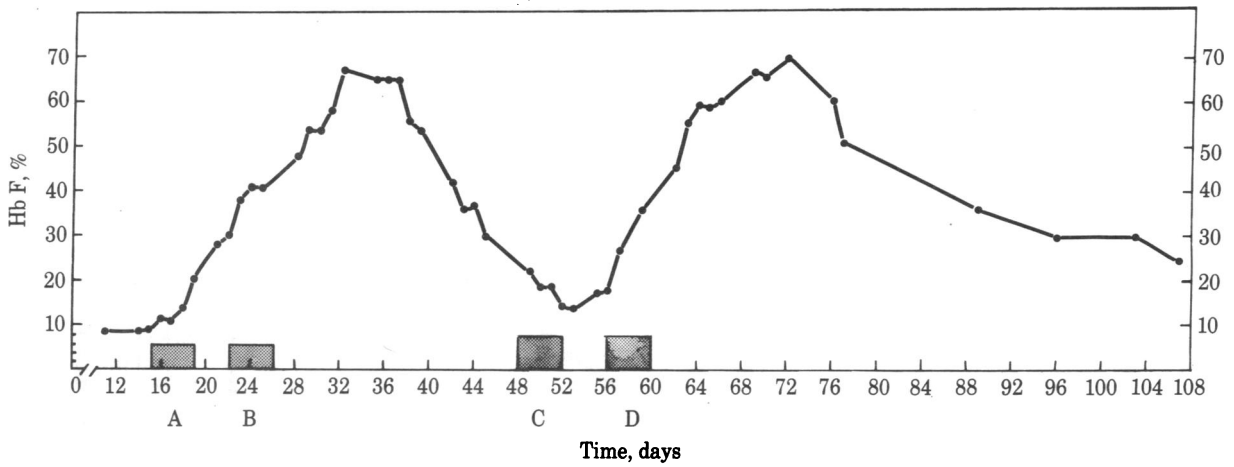

FIG. 1. Changes of Hb F levels in baboons rendered anemic by bleeding and then injected with 5-azaC: A, 6 mg/kg per day on days 15–19; B, 6 mg/kg per day on days 22–26; C, 8 mg/kg per day on days 49–53; D, 8 mg/kg per day on days 56–60.

(in which the cytosine ring is normal and the pentose is modified) to determine its effect on Hb F production. The regimen was similar to that for 5-azaC (3 mg/kg daily each weekday for 2 weeks). This animal was subsequently treated with 5-azaC.

Thirteen animals were used in this study. All animals except the one that was not bled received adequate amounts of iron dextran, folate, and vitamin B-12.

RESULTS

During the period of bleeding alone (days 1–15), maximal Hb F levels in the high and low responders were 10% and 2%, respectively. Five to 7 days after the initiation of treatment with 5-azaC, Hb F levels started to increase and reached their peaks 5–7 days after discontinuation of the drug. These peak levels were 67% and 81% in the two high responders and 32% and 33% in the two low responders. They were maintained for 5–7 days even though 10–20% of the blood volume was removed daily with the intention of maintaining the PCV at approximately 20%. In the animal for which the Hb F levels are shown in Fig. 1 and Table 1, the PCV varied between 18% and 24% except that on each Monday after the 2-day rest period the PCV was regularly higher (up to 33%) with a corresponding decrease in the reticulocyte index.

The relative rates of γ and β chain synthesis measured by incorporation of [^{3}H]leucine into peripheral reticulocytes were directly proportional to the maximal peripheral blood levels of Hb F. The $\gamma/(\gamma + \beta)$ synthesis ratio was 0.71 and 0.85 in the two high responders and 0.4 and 0.39 in the two low responders. The maximal number of F-cells was found within 5–7 days after the initiation of the drug treatment and stayed at a plateau; the maximal level of Hb F was achieved later, suggesting that the major effect of 5-azaC on Hb F production is an increase in Hb F per F cell (Fig. 2). The cellular content of Hb F varied, so cells with high, low, or no Hb F content could be distinguished. The proportions of such cells were approximately 90%, 5%, and 5% in the high Hb F responders and 45%, 35%, and 20% in the low responders.

During the course of treatment the mean corpuscular volume in all animals increased from 77 ± 0.8 to 102 ± 2.5 fl and the mean corpuscular hemoglobin increased proportionally from 26.6 ± 0.45 to 34.5 ± 0.52 pg per cell. The total leukocyte count decreased from 6–10 × 10^3 to 2–4 × 10^3 per μl and the granulocytes were slightly more affected than lymphocytes. More severe leukopenia was observed in one animal which received 8 mg/kg (Table 1) in a futile attempt to achieve even higher Hb F levels. The dose–response tests suggested that 5-azaC treatment at 3–4 mg/kg leads to maximal Hb F production. The maximal Hb F level in a control animal that was not bled or treated with 5-azaC was only 3.5%, with a reticulocyte index of <0.5%. However, Hb F in the animal's reticulocytes was synthesized at a rate comparable to that of the anemic animals—$\gamma/(\gamma + \beta) = 0.4$.

Hydroxyurea alone did not increase Hb F synthesis or Hb F levels. When this drug was administered 30 min prior to each dose of 5-azaC, stimulation of Hb F producation was decreased by at least 70% compared to 5-azaC alone (Fig. 3). araC (3 mg/kg for 10 injections) given to one animal increased Hb F levels from 5% to 18%. When a course of 5-azaC (4 mg/kg each weekday for 10 days) was given afterward, Hb F levels increased to 42%.

DISCUSSION

The data are in keeping with the hypothesis that the increase in Hb F synthesis under the influence of 5-azaC is due to hy-

Table 1. Selected serial hematological values* in baboon 4005.

Day	PCV, %	Ret., %	MCH	MCV	Hb F, %	F cells, %	WBC
1	41	0.8	27	79	0.3	4	8.2
15†	23	26	31.1	103	8.4	28	7.8
16	23	22	33.2	103	8.9	57	8.4
19	17	24	34.7	108	13.7	70	8.9
23	24	21	35.5	111	28	62	5.8
26	20	18	36	108	40	82	9
30	26	11	36.8	109	47	81	7.6
34	20	27	34	106	67	89	8.3
37‡	29	25	32	107	64	97	7.2
46	23	24	31.6	101	36	68	5.9
51‡	33	17	29.8	98.4	22	52	4.4
58	24	10	31.7	97	17	—	2.6
60	18	7.5	33.8	97	27	63	3.9
68§	22	13	32.9	99.7	59	—	4.8
72	27	7.6	33.1	103.8	66	83	4.8
75	37	8.2	30.7	94	61	—	3.7
76	—	—	30.4	94.6	—	—	3.3
89	43	1	29.8	89.4	36	71	4.4
103	42	1.2	27.7	86.8	30	61	8.9

* Ret., reticulocytes, %; MCH, mean corpuscular Hb, pg; MCV, mean corpuscular volume, μm^3; WBC, leukocytes, no./μl.
† Start of treatment with 5-azaC: 6 mg/kg per day on days 15–19 and 22–26; 8 mg/kg per day on days 49–53 and 56–60.
‡ Day after weekend.
§ Daily bleedings (days 1–68) discontinued.

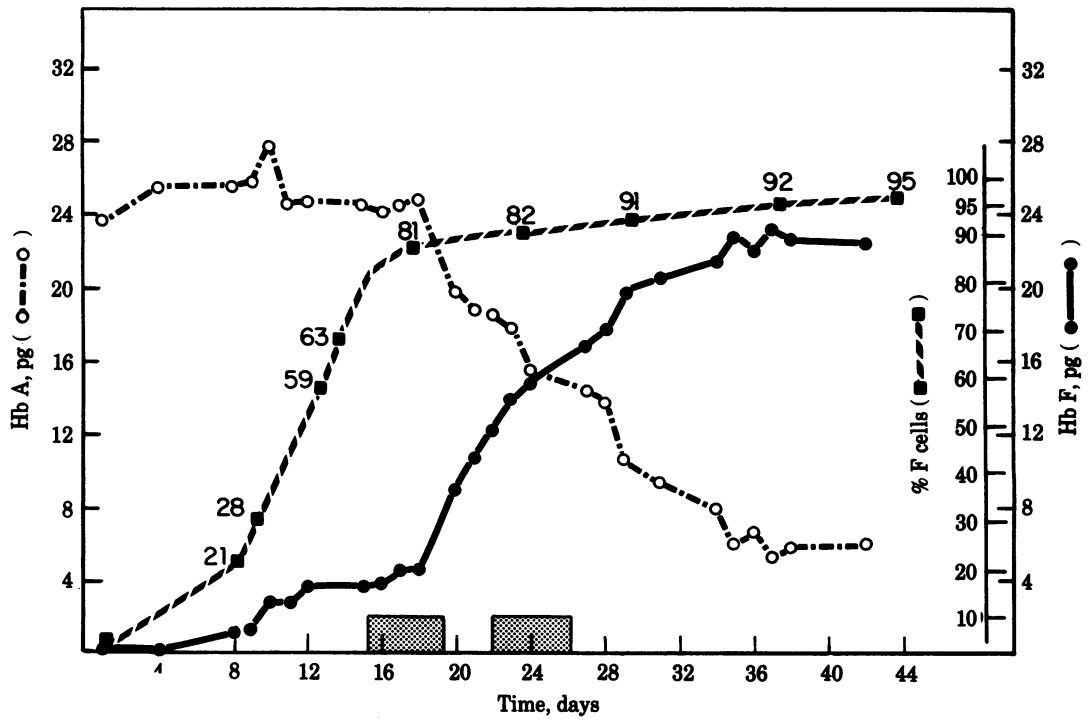

FIG. 2. Calculated mean corpuscular Hb values for Hb A and Hb F during treatment with 5-azaC (6 mg/kg per day) (rectangles) of a high responder adult baboon. Percentages of F cells are also shown. Note the rapid increase in the percentage of F cells with bleeding alone, followed by the increase in the amount of Hb F per erythrocyte.

pomethylation. Definitive proof must wait for restriction endonuclease analysis of DNA extracted from the marrow erythroid cells of treated and untreated animals, but the following facts are in favor of this mechanism of action. First, several studies have demonstrated that hypomethylation of DNA due to incorporation of 5-azaC results in gene expression (14–16). Second, hydroxyurea, which inhibits the enzymatic reduction of ribonucleoside diphosphates and therefore interferes with DNA synthesis, does not enhance Hb F production. Moreover, this drug interferes with the action of 5-azaC on Hb F production, suggesting that 5-azaC must be incorporated into DNA to exert its effect. Third, araC, a cytidine analogue with a normal cytosine ring but the ribose replaced by arabinose, increases Hb F levels only slightly. This slight increase is probably caused by

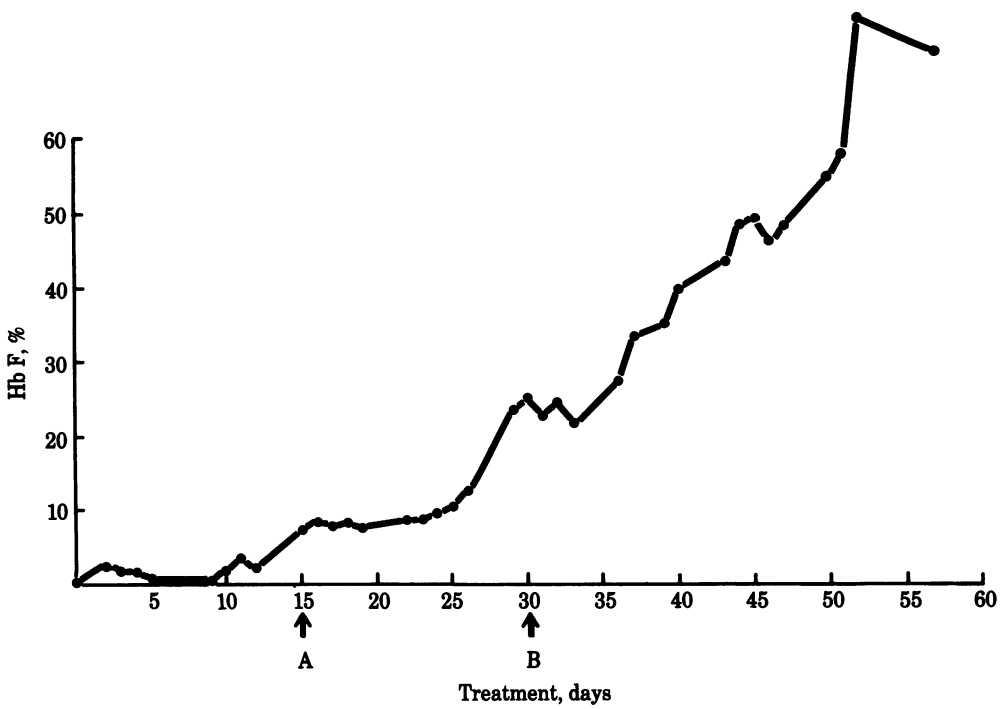

FIG. 3. Inhibition of the effect of 5-azaC on Hb F synthesis by hydroxyurea (see text). A, Treatment with 5-azaC plus hydroxyurea; B, treatment with 5-azaC alone.

the erythropoietic stress due to the combined effect of bleeding and the drug. In addition, after araC is incorporated into DNA, it may be slightly less methylated than the normal nucleotide, as has been suggested by G. P. Beardsley (personal communication).

Hypomethylation of the CpG sequences relevant to γ-globin gene expression in the adult might lead to a methylation pattern that is similar to that found in the fetus (5), thus allowing γ-globin gene expression. Because the relevant β-globin gene sequences in the adult are hypomethylated, 5-azaC should have no effect on β-globin production. The prevalence of γ-globin in the 5-azaC-treated adult animals could be explained by the known asynchrony of γ and β chain synthesis (17, 18). γ-Globin accumulates before β-globin, and the moderate increase in cell size in the 5-azaC treated animals (Table 1) could not provide the space for a normal amount of β-globin to be accommodated.

From the data it is possible to estimate that the maturation time of erythroid stem cells affected by 5-azaC is 6–12 days which is consistent with the assumption that the modification of the DNA takes place in all erythropoietic stem cells and promotes γ-globin gene expression. The previously described (7) stimulation of Hb F synthesis by erythropoietic stress may have a similar molecular basis, in the sense that methylation may occur during normal maturation, and rapid maturation due to stress erythropoiesis may be associated with hypomethylation. In this respect it is interesting that neither erythropoietic stress nor treatment with 5-azaC obliterates the genetic difference between low and high Hb F responders. Thus, this genetic difference must involve mechanisms other than differential methylation.

The demonstrated effect of 5-azaC may have therapeutic implications if similar Hb F increases could be achieved in man—e.g., in sickle cell anemia it might be expected that a high mean corpuscular hemaglobin value for Hb F under the influence of the drug would inhibit sickling (19). The use of a drug for this purpose, however, must have an acceptable risk/benefit ratio and, therefore, a myelosuppressive agent as 5-azaC is not suitable for this purpose unless it is possible to modify its structure so that its effect on γ-globin gene expression can be separated from cytotoxic effects.

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The Influence of Ethnicity on Warfarin Dosage Requirement

Mai-Trang N Dang, Julie Hambleton, and Steven R Kayser

BACKGROUND: The optimal dose of warfarin varies among individuals, and the prediction of a maintenance dose is difficult. Ethnicity has been reported to influence warfarin dosing.

OBJECTIVE: To quantitate the influence of ethnicity on warfarin dose requirement.

METHODS: We conducted a retrospective cohort study at a university anticoagulation clinic to evaluate the influence of ethnicity on warfarin dose. Inclusion criteria included age ≥18 years, target international normalized ratio (INR) 2–3, and warfarin management within the clinic for ≥3 months with a minimum of 5 clinic visits. We collected clinical and demographic data including age, gender, weight, ethnicity, disease states, concomitant medications, indication, weekly warfarin dosage, and INR. To assess potential confounders, multivariate, repeated-measures regression analysis was used to identify and adjust for variables that may influence the maintenance dose of warfarin.

RESULTS: Of the 345 patients who met the inclusion criteria, 27% were Asian American, 6% Hispanic, 54% white, and 14% African American. The adjusted mean (95% CI) weekly warfarin doses for patients with an INR goal of 2 to 3 were Asian Americans 24 mg (21 to 27), Hispanics 31 mg (25 to 37), whites 36 mg (34 to 39), and African Americans 43 mg (39 to 47) ($p < 0.001$). Additional factors found to influence warfarin dose requirement included age, weight, concomitant use of amiodarone, and diagnosis of venous thromboembolism.

CONCLUSIONS: Warfarin dose requirements vary across ethnic groups even when adjusted for confounding factors, suggesting that genetic variation contributes to interpatient variability.

KEY WORDS: ethnic influence, polymorphism, warfarin.

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The optimal dose of warfarin required to achieve a therapeutic international normalized ratio (INR) varies among individuals, and the prediction of a maintenance dose is difficult. Previous studies have shown that age, diet, certain disease states, and certain medications influence warfarin requirements.[1-8] Ethnic background and the presence of specific polymorphisms in the gene responsible for warfarin metabolism, CYP2C9, have also been implicated.[8-11]

A study evaluating warfarin dosing among Asian (patients from the Far East were excluded), Afro-Caribbean, and white patients found the average warfarin dose to be highest in Afro-Caribbeans and lowest in white patients.[10] These results differ from clinical observations and previous studies showing that Asians from the Far East require lower doses than white patients. Two studies demonstrated that the average daily warfarin dose required to achieve an INR of 2.0–2.5 in Chinese patients living in China ranged from 3.1 to 3.3 mg.[9,11] These studies did not, however, control for confounding variables and did not compare their Chinese cohort with a matched white or other ethnic control group. African American ethnicity has been identified as an independent predictor associated with a warfarin re-

Author information provided at the end of the text.

This study was presented as an abstract at the American Society of Hematology meeting, Philadelphia, PA, December 2002.

quirement >5 mg/day.[8] However, this study had a small sample size and limited ethnic group variation.

The primary objective of our study was to measure the influence of ethnicity on warfarin dose requirement. The study compared the mean warfarin dose among 4 ethnic groups after adjustment for confounding variables.

Methods

DESIGN

This retrospective study was conducted at a university hospital anticoagulation clinic and was approved by the institutional review board. Patients who attended the clinic between January 1, 2000, and September 27, 2001, were included if they met the following eligibility criteria: age ≥18 years, target INR 2–3, and warfarin management within the study clinic for ≥3 months with a minimum of 5 clinic visits. Patients were excluded if they were concomitantly taking antibiotics or antifungal agents.

Medical records were reviewed for age, gender, weight, ethnicity, comorbid disease states, concomitant medications, indication for warfarin therapy, weekly warfarin dose in milligrams, duration of warfarin therapy in the clinic, dietary habits, and relevant laboratory measurements. We evaluated consumption of foods known to contain large quantities of vitamin K by history only (vitamin K was not measured). Patients' ethnicity was obtained from the hospital computer system or medical records and categorized as white, African American, Asian American, or Hispanic.

Factors evaluated as potential confounders included age, gender, ethnicity, weight, indication for warfarin therapy, comorbid disease states, diet, and concomitant medications. Comorbid diseases included congestive heart failure, thyroid disease, liver disease (defined as liver function test results exceeding the normal ranges), acute febrile illnesses, renal disease, and malignancy. Additional chronic conditions evaluated were diabetes, hypertension, hyperlipidemia, and coronary artery disease. Smoking, unusual dietary habits, and excessive alcohol intake were also evaluated. Medications assessed had an interaction level of significance of 1, 2, or 3 according to Drug Interaction Facts.[12] The potentiators or inhibitors of warfarin's effect included amiodarone, phenytoin, barbiturates, carbamazepine, rifampin, thyroid supplements, cholesterol-binding resins, nonsteroidal antiinflammatory drugs (NSAIDs), aspirin, cimetidine, vitamin E, hydroxymethylglutaryl coenzyme (HMG-CoA) reductase inhibitors, and fibric acid derivatives. Additional medications evaluated included fluvoxamine and herbal supplements.

STATISTICAL ANALYSIS

Simple comparisons were performed using the Pearson χ^2 test for discrete outcomes and one-factor ANOVA for continuous outcomes. To assess potential confounders and accommodate repeated measurements, multivariate, repeated-measures regression analysis was used to adjust for variables that were known or possibly could influence the maintenance dose of warfarin. These variables included race, age, gender, weight, congestive heart failure, liver disease, deep venous thrombosis (DVT), thyroid disorder, and the use of amiodarone, NSAIDs, aspirin, phenytoin, barbiturates, carbamazepine, rifampin, resins, thyroid medications, vitamin E, HMG-CoA reductase inhibitors, and fibric acid. None of the subjects were taking cimetidine or fluvoxamine. Variables that had an insignificant number of subjects with the disease were not adjusted for in the analysis.

Log dose of warfarin was used in the multivariate regression to calculate the percent change in warfarin dose attributed to the possible confounders. Least-squares means calculated from the regression model and *t* statistics were used to compare the weekly warfarin dose for each ethnic and age group. Data are presented as mean ± SD or 95% CI and prevalence. All analyses were performed by Statistical Analysis System software, version 8.2. A 2-sided p value of <0.05 was considered statistically significant.

Results

Three hundred forty-five records met the inclusion criteria and were available for analysis. Table 1 summarizes the patients' characteristics at the time of data collection. The proportion of men to women was not significantly different among the ethnic groups. The mean age and weights were significantly different, and both were adjusted for in the multivariate regression analysis.

Table 1. Comparison of Patient Characteristics

Variables	Asian	Hispanic	White	African American	p Value
Demographics					
patients, n (%)	94 (27)	19 (6)	185 (54)	47 (14)	
age, mean ± SD (y)	67.7 ± 13.9	75.2 ± 8.1	67.3 ± 15.9	61.1 ± 18.9	0.007
weight, mean ± SD (kg)	66.1 ± 12.9	72.7 ± 17.6	79.0 ± 20.5	90.6 ± 22.9	<0.001
Indications, n (%)[a]					
atrial fibrillation	67 (71)	11 (58)	109 (59)	20 (43)	0.01
DVT	5 (5)	3 (16)	39 (21)	12 (26)	0.003
pulmonary embolism	4 (4)	2 (11)	24 (13)	9 (19)	0.04
TIA/stroke	23 (24)	6 (32)	25 (14)	12 (26)	0.03
↓LVEF	0	1 (5)	0	3 (6)	0.001
valve replacement	2 (2)	1 (5)	1 (1)	0	0.19
Comorbid diseases, n (%)[b]					
diabetes mellitus	26 (28)	4 (21)	24 (13)	13 (28)	0.01
hypertension	62 (66)	12 (63)	99 (54)	31 (66)	0.15
congestive heart failure	31 (33)	9 (47)	57 (31)	14 (30)	0.51
coronary artery disease	30 (32)	5 (26)	40 (22)	11 (23)	0.31
hyperlipidemia	48 (51)	9 (47)	76 (41)	17 (36)	0.29
hypothyroidism	18 (19)	2 (11)	40 (22)	1 (2)	0.01
liver disease	1 (1)	2 (11)	4 (2)	3 (6)	0.06
renal disease	15 (16)	0	19 (10)	9 (19)	0.09
malignancy	12 (13)	2 (11)	28 (15)	1 (2)	0.12

DVT = deep venous thrombosis; LVEF = left ventricular ejection fraction; TIA = transient ischemic attack.
[a]Other indications were Factor V Leiden, pulmonary hypertension, commissurotomy, ischemic heart disease, and atrial thrombus (all p > 0.05).
[b]Additional comorbid diseases examined were hyperthyroidism, epilepsy, smoking, excessive alcohol use, and peripheral vascular disease (all p > 0.05).

The mean duration of time patients were seen in the clinic varied from 2.3 to 2.7 years ($p = 0.68$). The number of INRs obtained per year and the percentage of therapeutic INRs were not significantly different among the ethnic groups. The prevalence of atrial fibrillation and transient ischemic attack were both significantly different among the ethnic groups; however, they have not been implicated in altering warfarin requirements independent of potential drug interactions. Other indications that were found to differ significantly included DVT ($p = 0.003$) and a decreased left ventricular ejection fraction ($p < 0.001$). Multivariate regression analysis adjusted for these variables.

Other differences in patient characteristics were comorbid disease states. Diabetes and hypothyroidism were significantly different (both $p = 0.01$). However, all patients with hypothyroidism were taking stable thyroid hormone supplements, and this variable was adjusted for in the multivariate regression model. All the medications assessed, including mean amiodarone dose, were not significantly different among the ethnic groups.

The result of the multivariate regression analysis demonstrated that ethnic background was an independent predictor of warfarin requirement ($p < 0.001$, Figure 1). The adjusted mean weekly warfarin doses (95% CI) for an INR goal of 2–3 were Asian Americans 24 mg (21 to 27), Hispanics 31 mg (25 to 37), whites 36 mg (34 to 39), and African Americans 43 mg (39 to 47) ($p < 0.001$). All of the mean weekly warfarin doses were significantly different when each ethnic group was compared with each other with the exception of Asians with Hispanics and Hispanics with whites.

Within 94 subjects in the Asian group, we analyzed the mean warfarin dose among the different ethnic groups. The distribution was as follows: 53 (56%) Chinese, 25 (27%) Filipinos, 4 (4%) Koreans, and 12 (13%) other Asians. The results did not show a significant difference between the mean weekly warfarin doses among the groups.

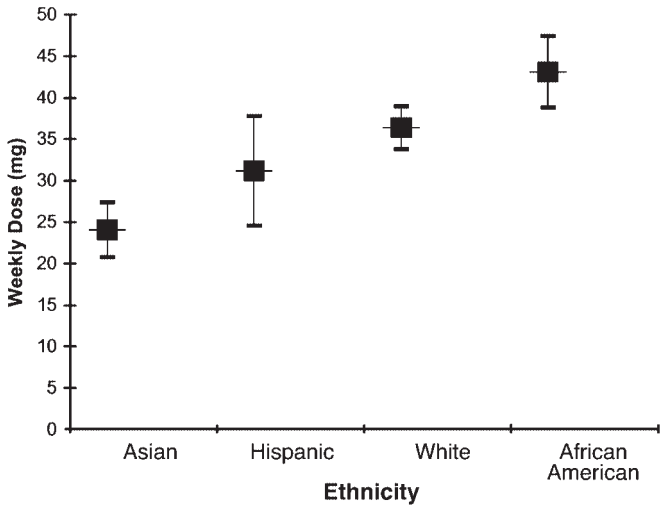

Figure 1. Adjusted mean weekly warfarin dose (95% CI) among different ethnic groups. The doses were adjusted for race, age, gender, weight, congestive heart failure, liver disease, deep venous thrombosis, hypothyroidism, and use of amiodarone, nonsteroidal antiinflammatory drugs, aspirin, phenytoin, barbiturates, carbamazepine, rifampin, thyroid supplements, resins, vitamin E, hydroxymethylglutaryl coenzyme A reductase inhibitors, and fibric acid ($p < 0.001$).

With increasing age, the dosage requirement for warfarin decreased ($p < 0.001$). In all, the average dose of warfarin decreased by 47% from the ages of 30–39 to ≥80 years. The influence of age appears to be consistent among ethnic groups as well. After adjusting for variables, weight was also found to significantly influence warfarin dose ($p = 0.001$). With each increase of 10 kg in weight, the dose requirement increased by 1 mg/wk.

Amiodarone and indication of DVT independently influenced warfarin dose requirement. Patients receiving amiodarone (mean dose 206 ± 72 mg) required 18% lower warfarin doses ($p = 0.005$). Patients with an indication of DVT required 21% higher warfarin doses than those who did not have the indication ($p = 0.01$).

Discussion

Consistent with findings from other studies, our study demonstrated that advanced age, use of amiodarone, and indication of DVT influence warfarin requirements.[1-4,6,7] Weight affected warfarin requirements in our patient cohort; however, this effect may be small and is inconsistent across other studies.[8-10] Our study further extends the current observation that ethnicity affects warfarin dose requirements independent of previously identified variables.

There are several limitations to this study. First, it was retrospective. In addition, some of the variables controlled for may be inherent in certain ethnic groups, but these confounders were adjusted for by the multivariate regression and ethnicity was still found to significantly influence warfarin dosage requirements. Third, despite efforts to ensure weekly dose calculation at a time when patients were at their target INR, there is a possibility that some INRs may have been outside this range. We did not exclude patients with an INR outside of the therapeutic range because many patients on a stable warfarin regimen will sometimes have INRs that fluctuate outside the narrow therapeutic range, and many times clinicians may not change the warfarin dose. The percentage of time that our patients were within the therapeutic range is consistent with reports from other anticoagulation clinics. Lastly, vitamin K intake was not measured, although patients were interviewed at each clinic visit for variations in diet specifically with respect to vitamin K content. One trial measured plasma vitamin K levels in patients taking warfarin and, even with direct measurement, the investigators concluded that there was no relationship between plasma vitamin K levels and warfarin dose.[13] We did not detect by history any difference in dietary vitamin K use among the patient groups, although this remains a potential limitation.

The cause of the observed difference in warfarin dose requirements among the ethnic groups has several possible explanations. Differential protein binding has been proposed to

contribute to the variability in drug response. Zhou et al.[14] investigated the binding of various constituents in plasma to albumin and α-acid glycoprotein in healthy Chinese and white subjects.[14] The results from this study showed that the unbound fraction of warfarin binding to albumin was similar in both groups. No significant difference in plasma albumin concentration was noted among the groups. Although protein binding may be an important factor in initial warfarin response, it is not a factor with chronic dosing. Based upon the investigators' observations, it is not likely to be the basis for either a difference in the initial dose or the steady-state maintenance warfarin dose among ethnic groups. Patients may have increased affinity for warfarin and/or are intrinsically more sensitive to the action of warfarin; however, this has not yet been tested.

Genetic differences in drug metabolizing capacity across ethnic groups may account for the variable response observed with warfarin. Studies investigating genetic polymorphisms of CYP2C9 have demonstrated an association between different allelic variants and warfarin sensitivity.[15,16] Numerous studies have investigated the prevalence of CYP2C9 polymorphisms among different ethnic groups.[17-22]

The wild-type allele, *2C9*1*, is the most common, with a frequency of 78–86% in whites, 69.4% in Hispanics, 95–98% in Asians, and 95–99% in African Americans. The *2C9*2* allele is observed in about 20% of whites and 1% of African Americans and has not been detected in Chinese or Japanese.[23] The *2C9*3* polymorphism has a low prevalence across all ethnic groups: 3.7–9.2% in whites, 16.2% in Hispanics, 0.5–1.25% in African Americans, and 1.7–2.6% in Asians.[16-21] The *2C9*2* and *2C9*3* allelic variants have been associated with increased warfarin sensitivity and, compared with *2C9*1*, *2C9*3* has 5% of the metabolizing capacity.[15,16] Based on our observations of an increased sensitivity to warfarin in Asian patients and the very low frequency of the allelic variants associated with warfarin sensitivity, the *2C9*2* and *2C9*3* alleles are unlikely to be the explanation for these ethnic differences in dose requirement.

A novel and prevalent polymorphism in the Chinese sample was recently described. Chinese patients with a possible polymorphism at exon 4 in the CYP2C9 gene appeared to have reduced warfarin requirements, but these results have been refuted.[24,25] Studies of polymorphisms in the promoter region of CYP2C9 suggest a potential mechanism for the lower dose requirement in Asians.[26] Complete CYP2C9 gene sequencing and identification of haplotypes will be critical to fully understanding the role of CYP2C9 in patient variability. Information from ongoing studies and the results of our analysis may provide the basis for a more comprehensive evaluation of the presence of genetic polymorphisms among different ethnic groups.

Conclusions

This study quantitates the influence of ethnicity on warfarin dose requirement independent of other well-established variables such as age, weight, certain medications, and indication of DVT. While the exact mechanism by which ethnicity influences warfarin requirement has not been established, it is recommended that patients who are of Asian ethnicity should be started on lower doses (~50%) than white or African American patients. A more accurate prediction of initial warfarin requirements may potentially decrease the rates of hemorrhagic and thrombotic complications with the use of this narrow therapeutic index drug. This is especially true since the incidence of hemorrhage is increased during the initial stages of warfarin therapy.

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Ethnic Differences in Cardiovascular Drug Response
Potential Contribution of Pharmacogenetics

Julie A. Johnson, PharmD

In the early 1980s, clinical differences in response to the blood pressure (BP)–lowering effects of β-blockers and, to a lesser extent, diuretics were noted between ethnic groups. The most convincing evidence at that time came from a Veterans Affairs (VA) Cooperative Trial,[1] which, along with other smaller studies, suggested that whites (those of European ancestry) had a better antihypertensive response to β-blockers than blacks (those of African ancestry), whereas blacks had a slight better response to diuretics than whites. Shortly after the first angiotensin-converting enzyme (ACE) inhibitor was approved in the mid-1980s, it was also recognized that whites responded more favorably to ACE inhibitors than did blacks. Over time, these differences in response became well accepted, such that ethnicity began to be used in helping to guide selection of antihypertensive drug therapy.[2,3] Although the ethnic differences in response between β-blockers and ACE inhibitors in hypertension are perhaps the mostly widely recognized examples of ethnic differences in response to cardiovascular drugs, there are others.

Pharmacogenetics is a field that seeks to unravel the genetic underpinnings of variable drug responses.[4] Given the recognized ethnic differences in drug responses and the fact that many genetic polymorphisms differ in frequency on the basis of ethnicity/ancestry, questions about whether pharmacogenetics may also lead to an understanding of the ethnic differences in drug response are not surprising. The present review will summarize the most widely recognized examples of cardiovascular drugs with differential response by ethnicity and the evidence that pharmacogenetics data may aid in our understanding of these differences. Given that there are many examples in the literature of genetic associations that are not replicated, the pharmacogenetic examples discussed herein will come from those for which there is some evidence of replication or for which there have been multiple negative findings.

In light of the socially charged issues that surround race and genetics, we will typically refer to groups either as ethnic groups (meaning groups who may have similar ancestral origins and who share certain social or cultural practices) or will refer to continental ancestry, referring to the 3 major continental populations from which the human population mainly derives (namely, European, African, and Asian ancestry).

Ethnic Differences in Response to Warfarin Therapy

Ethnic differences in the warfarin dose required for an international normalized ratio (INR) between 2 and 3 are well documented in the literature but do not appear to be widely appreciated by clinicians. For example, the anticoagulation consensus guidelines that relate specifically to warfarin do not mention the influence of ethnicity on the typical maintenance dose,[5] a fact that may result from trials conducted predominantly in white populations. Figure 1 depicts average warfarin dose requirements for Asians, Hispanics, whites, and blacks to maintain an INR of 2 to 3.[6] Although these data were derived from a relatively small sample, average daily doses of 3.4 mg in Asians, 5.1 mg in whites, and 6.1 mg in blacks are representative of the literature for these ethnic groups. Given that most dosing algorithms recommend initiating therapy at 5 mg daily, it is apparent from Figure 1 that this is a reasonable estimate of the starting dose in whites but likely an excessive dose in Asians and an inadequate dose in blacks. The lower dose requirement in Asians was sufficiently recognized to warrant special notation in US Food and Drug Administration (FDA)–approved labeling for warfarin, which indicates requirements for a lower dose in Asians.[7] Although some would argue that initiation of therapy with an inappropriate dose will be corrected quickly on the basis of close monitoring of INR, data clearly suggest the risk of bleeding is highest in the first 30 days of therapy, when the appropriate dose is typically still being determined.[8] This would suggest that more accurate initial dosing may have the potential to reduce the early risk of bleeding.

In addition to differences in dose, there are questions about whether the risks of warfarin therapy also differ by ethnicity. The large trials that established an INR range of 2 to 3 to balance the benefits (reduced thromboembolic events) with the risks (bleeding) of warfarin therapy were conducted almost exclusively in whites. Thus, it is not clear whether this is the most appropriate INR range across ethnic groups, although some data suggest it may not be in Asians. For example, in a study of 563 Taiwanese patients with mechanical valve replacements (for whom the usual INR range is 2.5 to 3.5), investigators found the risks of thromboembolism were not different for those with an INR >2 versus <2.[9] In

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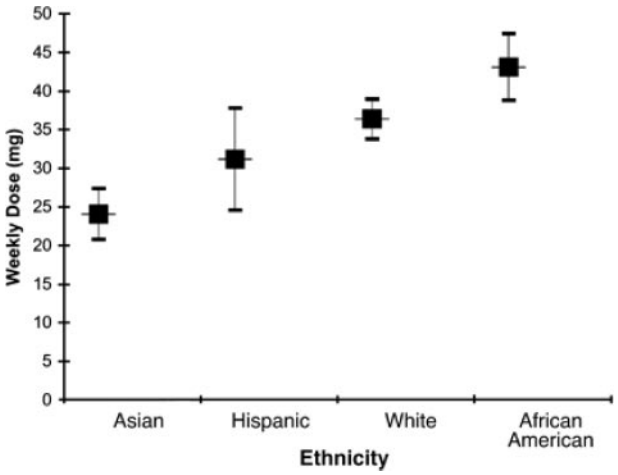

Figure 1. Average warfarin dose requirements, by ethnicity, to maintain a therapeutic INR.[2,3] Reproduced from Dang et al,[6] with permission from the *Annals of Pharmacotherpy*.

a study of 491 Chinese patients treated with warfarin, the INR associated with the lowest hemorrhagic and thromboembolic rate was 1.8 to 2.4.[10] These data suggest Asians may have greater thromboembolic protection at lower INRs than whites. Finally, in a study of 667 Japanese nonvalvular atrial fibrillation patients studied for 1 year, INR ≥2.27 was associated with an OR of 4.33 (95% CI 1.30 to 14.39) for major bleeding. Furthermore, despite low-dose warfarin therapy (target INR 1.6 to 2.6), the rate of major bleeding and intracranial hemorrhage was similar to the rate observed in Western populations with full-dose anticoagulation (target INR 2 to 3) and approximately double the rate observed in Western populations for low-intensity warfarin therapy.[11] Combined, these data suggest that Asians might require a lower INR for protection from thromboembolism and might be at increased risk of bleeding at lower INRs.

Warfarin Pharmacogenetics

Among cardiovascular drugs, warfarin has the strongest pharmacogenetics data, which may also help explain ethnic differences in dose requirements for a stable INR. Two genes have been clearly associated with a variable warfarin dose: those encoding the major enzyme responsible for the metabolism of warfarin (cytochrome P450 2C9, *CYP2C9*) and the protein on which warfarin exerts its pharmacological effect (vitamin K epoxide reductase, *VKORC1*). The first report of genetic association with warfarin dose and *CYP2C9* genotype was in 1999,[12] and numerous studies since that time have documented this association across a variety of ethnic populations (see reviews by Wadelius and Pirmohamed[13] and Sanderson et al[14]). Specifically, there are 2 polymorphisms, commonly called CYP2C9*2 and CYP2C9*3, both of which reduce the normal metabolic activity of the enzyme, although the *3 polymorphism does so to a greater extent than the *2 polymorphism. In a 2005 meta-analysis, which included 2775 patients and 8 different studies that related the polymorphisms to warfarin dose, the analysis suggested that carriers of at least 1 variant copy of the *2 allele required 0.85 mg less of warfarin daily (95% CI −1.11 to −0.60 mg), and those carrying at least 1 copy of the *3 allele required 1.92 mg less of warfarin daily (95% CI −2.47 to −1.37 mg).[14] Several studies have also documented that individuals with *CYP2C9* variant alleles require a longer period of time to achieve a stable dose and are at increased bleeding risk, particularly during the period of therapy initiation (ie, first 1 to 3 months).[12,14–16] Data on the influence of *CYP2C9* variants are available from multiple populations in the United States, Europe, and Asia, and all consistently show a genetic association with *CYP2C9* polymorphisms. What differs is the frequency of the polymorphisms and thus their overall impact in that ethnic population. Table 1 depicts allele frequencies for the *CYP2C9* variant alleles and shows there are clear differences by ethnicity. Specifically, variant alleles for *CYP2C9* are much more common in whites than other groups; thus, at a population level, the impact of *CYP2C9* variants on warfarin dose is greater in whites. This may help to explain the slightly lower doses in whites versus blacks but does not explain the very low doses typically required by Asians.

Table 1. Ethnic Differences in Variant Allele Frequencies for Genes Important to Variable Warfarin Dose/Response (*CYP2C9* and *VKORC1*)

Variant	Whites	Blacks	Asians
CYP2C9*2	8% to 18%	Rare	Rare
CYP2C9*3	5% to 13%	1% to 2%	2% to 5%
Others†	Rare/absent	2% to 4%	Rare/absent
VKORC1 variant‡	35% to 45%	8% to 10%	90% to 95%

Data derived from various sources.[14,16–18]

†Others includes CYP2C9*4, *5, *6, and *11.

‡The studies have included a variety of VKORC1 SNPs, which, due to strong linkage disequilibrium, have similar or identical allele frequencies, and all show significant association with warfarin dose. Most commonly studied are 3673 G>A (also known as −1639; rs9923231), 6484 C>T (also known as 1173; rs9934438), and 6853 G>C (rs8050894). Depicted here are typical variant allele frequencies for −1639 and the SNPs in strong linkage disequilibrium with it.

Differing warfarin sensitivities by ethnicity are perhaps better explained by variant alleles in *VKORC1*. A number of different polymorphisms have been studied in this gene, and evidence currently points to a promoter polymorphism (referred to in the literature as 3673 G>A or −1639 G>A) as the most likely candidate for the functional polymorphism.[19,20] Importantly, many different polymorphisms have been studied, and because of a high degree of linkage disequilibrium (inheritance of single-nucleotide polymorphisms [SNPs] together) between these SNPs in whites and Asians, the various SNPs tested all gave similar genetic associations. However, as with many other genes, the degree of linkage disequilibrium in *VKORC1* is lower in blacks than in other groups. In analyses in our laboratory of a variety of *VKORC1* SNPs, only 3673 and 6484 were significantly associated with warfarin dose in blacks, whereas numerous SNPs were associated with dose in whites. This is explained by high levels of linkage disequilibrium across numerous SNPs in whites but only these 2 SNPs in blacks. This emphasizes the importance of studying the functional polymorphism, because reliance on linkage disequilibrium between SNPs can be problematic across different ancestral

populations. Table 1 also provides a comparison by ethnicity for the presumed functional *VKORC1* polymorphism and reveals striking differences, such that the "variant" (ie, less common allele) in whites and blacks (with approximate frequencies of 45% and 10%, respectively) is the major allele in Asians, with a frequency of 90% to 95%.[17]

To date, there have been >30 studies published on the genetic association between *VKORC1* SNPs and warfarin dose, and all have shown a significant association, with the variant allele being associated with a lower warfarin dose.[13,19–26] These studies have included numerous white populations from the United States, Europe, and Israel, along with Japanese, Chinese, Indians, and Malays. In whites, across a variety of studies, the average dose for GG homozygotes (using −1639 as the reference) was 6.1 mg daily, whereas those with a GA genotype required 4.5 mg daily, and AA homozygotes required 3.0 mg daily. Among Asians, doses for GG and GA have often not been reported separately (owing to low G allele frequency), but across studies, AA homozygotes required 2.8 mg daily, similar to the dose required by whites with the AA genotype. In the single study with a reasonably sized black cohort, daily dose requirements for GG, GA, and AA genotypes were 5.7, 4.5, and 3.1 mg, respectively, nearly identical to that in whites.[21] Given that most blacks have the GG genotype and most Asians the AA genotype, these data suggest genetics may contribute substantially to the ethnic differences in dose.

Taken together, there is little doubt that genetic variability helps explain differences in warfarin dose requirements, particularly the *VKORC1* polymorphisms. Numerous different investigative groups have attempted to determine the amount of variability in warfarin dose that can be explained by genetic, demographic, and clinical factors. These studies suggest that between 30% and 60% of warfarin dose variability can be explained, with genetic factors responsible for explaining approximately two thirds of that variability. Clinical/demographic factors that have also been associated consistently with warfarin dose variability are age (reduced dose with increasing age), body size (increased dose with increased body size, assessed as body surface area, body mass index, or weight), and, in most studies, smoking status and interacting drugs. Given the well-known effect of high-content vitamin K foods on warfarin dose requirements, it is also possible that dietary differences between ethnic groups contribute to differences in warfarin sensitivity. It is also possible, although not tested to date, that there may be significant gene-diet interaction, particularly with *VKORC1* or other genes in the vitamin K pathway, that may also contribute to variability and might differ by ethnicity. Thus, in addition to genotype, there are a variety of other demographic, clinical, and environmental factors that may contribute to ethnic differences in warfarin dose requirements.

To advance the clinical translation of these findings, several groups have suggested warfarin dosing equations that incorporate genetic and nongenetic factors, some of which have been tested prospectively in small cohorts.[22,18,27–29] Two studies have tested prospectively a genotype-guided versus usual-dosing control group, with 1 study considering only *CYP2C9*[30] and the other considering both *CYP2C9* and *VKORC1*.[31] Both studies were relatively small (≈200 subjects each) and had mixed results regarding significant differences in specified outcomes between genotype-guided versus usual-care approaches. However, these studies and others clearly support the need for an adequately powered randomized clinical trial.

One of the challenges regarding clinical use of warfarin pharmacogenetic information is the lack of availability of a dosing algorithm/equation that has relevance across various geographic and ethnic groups. On the basis of this and other issues, investigative teams with warfarin pharmacogenetics data have shared their data in a common database, with the primary goal of defining a warfarin pharmacogenetics dosing equation with validity across the globe. It is anticipated that this dosing equation will incorporate information not only on *VKORC1* and *CYP2C9* genotypes but also on various clinical and demographic factors that influence warfarin dose requirements. The group, called the International Warfarin Pharmacogenetics Consortium, comprises 21 research groups from 11 countries and 4 continents, and combined, they have contributed warfarin genotype and phenotype data on nearly 6000 individuals, with all 3 major ethnic groups well represented. After publication of the first report from this group, all data will be made publicly available on a World Wide Web site for the Pharmacogenetics and Pharmacogenomics Knowledge Base (www.pharmgkb.org). An additional aim of the International Warfarin Pharmacogenetics Consortium is to test questions relating to genetic associations and ethnicity, given that the combined group will have greater power than single-site studies to test a variety of hypotheses relating to ethnicity and warfarin pharmacogenetics.

Utilization of genetic information for warfarin dosing made headlines in both the medical and lay press in the summer of 2007 when the FDA product labeling (package insert) for warfarin was changed to include suggestions on (but not require) the use of genetic information to guide early warfarin dosing. There is great controversy about whether these data are to the point that such clinical utilization is appropriate, because there have been only 2 small randomized prospective studies testing the prospective use of genetic information to guide warfarin dosing.[30,31] These questions will be addressed more comprehensively by a study from the National Heart, Lung, and Blood Institute, which will conduct a prospective clinical trial that tests genotype-guided warfarin dosing against usual-dose–initiation approaches. The study is intended to launch in late 2008 and last ≈18 months. This trial will not be powered to test (as a primary end point) for reductions in incidence of bleeding or prevention of thromboembolic events with the randomized dosing strategies. That the *CYP2C9* genotype is associated with bleeding risk seems clear, but it is not known whether prospective use of genetic information will reduce bleeding events. To the extent that some clinicians will judge reduced risk for bleeding to be the only meaningful end point for prospective warfarin pharmacogenetic testing, this may represent a long-term limitation of the data. Other clinicians will judge other end points to also be clinically meaningful (eg, time to stable INR or time to INR >4), and these should be well addressed by the planned trial. In the meantime, clinicians will be faced with deciding

Special Article

DIFFERING BIRTH WEIGHT AMONG INFANTS OF U.S.-BORN BLACKS, AFRICAN-BORN BLACKS, AND U.S.-BORN WHITES

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ABSTRACT

Background In the United States, the birth weights of infants of black women are lower than those of infants of white women. The extent to which the lower birth weights among blacks are related to social or genetic factors is unclear.

Methods We used vital records for 1980 through 1995 from Illinois to determine the distribution of birth weights among infants born to three groups of women — U.S.-born blacks, African-born blacks, and U.S.-born whites.

Results The mean birth weight of 44,046 infants of U.S.-born white women was 3446 g, that of 3135 infants of African-born black women was 3333 g, and that of 43,322 infants of U.S.-born black women was 3089 g. The incidence of low birth weight (weight less than 2500 g) was 13.2 percent among infants of U.S.-born black women and 7.1 percent among infants of African-born black women, as compared with 4.3 percent among infants of U.S.-born white women (relative risks, 3.1 and 1.6, respectively). Among the women at lowest risk (those 20 to 39 years old, with 12 years of education for themselves and their spouses, early prenatal care, gravida 2 or 3, and no previous fetal loss), the rate of low birth weight in infants of African-born black women (3.6 percent) was closer to the rate in infants of U.S.-born white women (2.4 percent), and the rate in infants of U.S.-born black women remained high (7.5 percent).

Conclusions The birth-weight patterns of infants of African-born black women and U.S.-born white women are more closely related to one another than to the birth weights of infants of U.S.-born black women. (N Engl J Med 1997;337:1209-14.)

DURING the past 40 years, epidemiologic research has elucidated many important associations between the sociodemographic characteristics of mothers and the birth weight of infants.[1-4] For example, the extremes of childbearing age,[1] cigarette smoking,[2] inadequate prenatal care,[3] urban poverty,[4] and black race[5] are well-documented risk factors for low birth weight. Other obstetrical risk factors account for part of the racial disparity in birth weights, but differences persist.[6-9]

Although the incidence of low birth weight decreases in both blacks and whites as the number of risk factors declines, the improvement is faster among whites, resulting in a wider birth-weight gap between blacks and whites among infants of low-risk women.[1,4] This has led some investigators to believe that genetic factors associated with race influence birth weight.[10-15] In the 1967 National Collaborative Perinatal Project, only 1 percent of the total variance in birth weight among 18,000 infants was accounted for by socioeconomic variables, leading the authors to conclude that "race behaves as a real biological variable in its effect on birth weight. This effect of race [is] presumably genetic."[10] The assumption that black women differ genetically from white women in their ability to bear normal or large infants persists in more recent studies of fetal growth,[13,16] one of which, for example, refers to "genetic factors affecting growth, such as neonatal sex and race."[16]

Few data have been published on the birth weights of infants born to African-born women in the United States. Most African Americans trace their origins to western Africa, where the slave trade flourished in the 17th and 18th centuries.[17,18] It is estimated that U.S. blacks derive about three quarters of their genetic heritage from West African ancestors and the remainder from Europeans.[18-21] To the extent that population differences in allele frequency underlie the observed differences in birth weight between blacks and whites in the United States, one would expect women of "pure" West African origin to bear smaller infants than comparable African Americans, considering the European genetic admixture in the latter. However, to our knowledge, no population of West African women delivering infants in the United States has been studied. We therefore undertook an analysis of racial differences in birth weight based on U.S.-born and African-born women giving birth in Illinois.

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METHODS

Study Population

We obtained data on the birth weights of singleton black and white infants born in Illinois and the birthplaces of their mothers, using birth-certificate tapes for 1980 through 1995 from the Illinois Department of Public Health. All the white infants studied had U.S.-born mothers and were not of Latino origin. The mothers of the black infants fell into two groups: women born in sub-Saharan Africa and those born in the United States. We selected random samples of the white and black U.S.-born women in order to have groups convenient for analysis; these groups included 2.5 percent of white births and 7.5 percent of black births.

Black women born in the Western Hemisphere but not in the United States (i.e., born in Canada, the Caribbean, or South America) were excluded from the study. Such designations of maternal origin were available for the period 1980 through 1988. During that period, birth records were coded with three separate fields: the mother's race, the mother's place of birth, and the mother's origin or descent. Women whose race was coded as "black," whose place of birth was coded as "not in Western Hemisphere," and whose origin or descent was coded as "Africa, excluding northern Africa" were considered to have immigrated from sub-Saharan Africa. According to the 1990 Census, 66 percent of African-born blacks living in Illinois for whom a sub-Saharan country of birth was recorded came from either Nigeria or Ghana.[22] From 1989 on, the variable indicating origin or descent was replaced by a variable specifically pertaining to Hispanic origin, but a new, detailed set of birthplace codes allowed us to identify births on the basis of the mother's country of birth. We therefore selected births from 1989 through 1995 in which the mother's birthplace was 1 of 17 present-day countries corresponding to the area from which African slaves originated in the 17th and 18th centuries.[18,20]

Analysis of Birth Weights

As a first step toward exploring the possible contribution of genetic factors to the racial disparity in outcomes of pregnancy, we compared the curves for the distribution of birth weight, the mean birth weights, and the rates of low birth weight (defined as the number of births of infants weighing less than 2500 g per 100 live births) of infants born to U.S.-born blacks, African-born blacks, and U.S.-born whites. In addition, we computed rates of moderately low (1500 to 2500 g) and very low (<1500 g) birth weight. Next, we determined the distribution of sociodemographic risk factors (the mother's age, education, and marital status, the trimester of first prenatal care, and the father's education) and reproductive risk factors (the overall number of pregnancies and whether there was a history of fetal loss or infant death) in the three groups of women. For the risk factors and outcomes, we calculated relative risks and 95 percent confidence intervals, using the infants of U.S.-born white women as the reference group.[23]

Because the three populations differed, we repeated the birth-weight comparisons after adjustment for differences in risk profiles. We did so in three ways. First, we compared each African-born mother with two similar U.S.-born women, one white and one black, who were matched for age, education, marital status, prenatal care, parity, and history of fetal loss. Second, we used the REG procedure (SAS, release 6.07, Cary, N.C.) to create a model showing birth weight as a function of all the risk factors for which data were available, except paternal education (data on that variable were missing for 20 percent of births) and prior loss of an infant (prevalence, <5 percent). We then estimated mean differences in birth weight among the three subgroups, both by subtracting intercept terms estimated in three subgroup-specific models and by modeling the subgroups two at a time, with ethnic status entered as a dichotomous dummy variable.[24] Third, we repeated the birth-weight analysis but limited it to subgroups of low-risk women defined according to social, demographic, and reproductive risk factors.

Our analysis used birth-certificate tapes from which the identifying information on the individual women and their infants had been removed. These data were provided by the Illinois Department of Health, which provides such "sterilized" birth tapes to researchers conducting epidemiologic studies.

TABLE 1. BIRTH-WEIGHT DATA IN ILLINOIS, 1980–1995, ACCORDING TO THE MOTHER'S RACE AND PLACE OF BIRTH.*

VARIABLE	SUBGROUP OF MOTHERS			RELATIVE RISK (95% CI) IN BLACK MOTHERS†	
	U.S.-BORN WHITES	AFRICAN-BORN BLACKS	U.S.-BORN BLACKS	AFRICAN-BORN	U.S.-BORN
Raw data					
No. of births	44,046	3135	43,322		
Mean birth weight (g)	3,446	3333	3,089		
Low birth weight (% of infants)	4.3	7.1	13.2	1.6 (1.4–1.9)	3.1 (2.9–3.2)
Moderately low	3.6	4.8	10.6	1.3 (1.1–1.6)	3.0 (2.8–3.1)
Very low	0.7	2.3	2.6	3.2 (2.5–4.1)	3.5 (3.1–4.0)
Matched cases‡					
No. of births	2,950	2950	2,950		
Mean birth weight (g)	3,475	3341	3,195		
Low birth weight (% of infants)	3.6	6.9	8.5	1.9 (1.5–2.4)	2.4 (1.9–2.9)
Moderately low	3.1	4.7	6.1	1.5 (1.2–2.0)	2.0 (1.5–2.5)
Very low	0.5	2.2	2.4	4.1 (2.4–7.0)	4.5 (2.6–7.7)

*Data on birth weight were missing for 19 infants (0.02 percent of the total). Low birth weight was defined as a weight of less than 2500 g, moderately low birth weight as a weight of 1500 to 2499 g, and very low birth weight as a weight of less than 1500 g.

†Relative risks shown are for the risk of low birth weight in the infants of women in the group shown as compared with the infants of U.S.-born white women. CI denotes confidence interval.

‡In this analysis, each African-born black woman was matched with one U.S.-born white woman and one U.S.-born black woman for age, marital status, education and spouse's education, prenatal care, parity, and the presence or absence of previous fetal loss.

RESULTS

The mean birth weight of the white infants was 3446 g, as compared with 3333 g for the infants of the African-born black women and 3089 g for the infants of the U.S.-born black women (Table 1). The proportion of very-low-birth-weight infants was similar for African-born blacks and U.S.-born blacks. Even though the infants born to African-born blacks had a slightly lower mean birth weight than the white infants, the overall distribution of birth weights was similar in the two groups and was different from that among the infants of U.S.-born blacks (Fig. 1).

Table 2 shows the distribution of selected risk factors in the three groups of women. The African-born black women delivered the highest proportion of infants who were their mothers' fourth or subsequent children and had the highest proportion of previous fetal and infant deaths. The U.S.-born black women were the youngest, the least likely to be married, the least well educated, and the most likely to have received prenatal care late or not at all. The white women surpassed both groups of black women with regard to only one risk factor — primigravidity.

When the infants of African-born black women were compared with those of U.S.-born women matched for the mother's age, marital status, education, prenatal care, parity, and prior fetal loss and the father's education, the differences between the groups narrowed somewhat, but their relation did not change (Table 1). With white infants as the reference group, the relative risks for low and moderately low birth weight were both significantly higher among infants of U.S.-born blacks than among infants of African-born blacks. However, the relative risk of very low birth weight was similar in the two groups of infants born to blacks.

To gain more insight into the relative importance of the risk factors in the three groups, we used multiple-regression analysis to study the changes in birth weight predicted by each factor. The models we constructed (Table 3) all showed a positive effect of being married (an increase of 60 to 124 g in predicted birth weight), having had one or two previous pregnancies (an increase of 29 to 50 g), and having no previous fetal loss (an increase of 19 to 55 g). Of the risk factors, only marital status had a statistically significant effect among the infants of African-born blacks.

On the basis of the multivariable models in Table 3, the birth weight of the infants of African-born blacks was 14 g less than that of the infants of U.S.-born whites after we controlled for risk factors. In another model, we looked at only the U.S.-born white women and the African-born black women, with race included as a dichotomous variable. In that analysis, the infants of the U.S.-born whites weighed 98 g more than the infants of the African-born blacks after adjustment for age, education, marital status, gravidity, prenatal care, and a history of fetal loss. In a similar model that included only women born in the United States, the white infants weighed 248 g more than the black infants after adjustment for the same six variables.

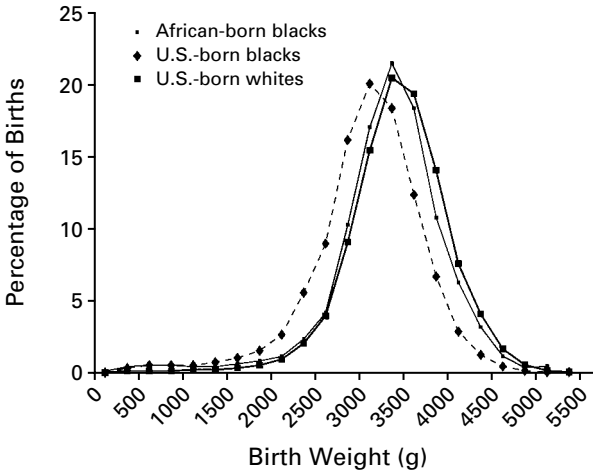

Figure 1. Distribution of Birth Weights among Infants of U.S.-Born White and Black Women and African-Born Black Women in Illinois, 1980–1995.

The calculation of frequencies was based on all singleton births in Illinois. The study population included the infants of 3135 black women born in sub-Saharan Africa, 43,322 black women born in the United States (a sample that included 7.5 percent of the total number of black women giving birth in Illinois), and 44,046 U.S.-born white women (2.5 percent of the total number of white women giving birth in Illinois).

Table 4 shows the mean birth weights and rates of low birth weight among infants born to the women at lowest risk — those 20 to 39 years of age who began their prenatal care in the first trimester, had at least 12 years of education, and were married to men who also had at least 12 years of education. Sixty-six percent of the white women fit this profile, as compared with 50 percent of the African-born black women and 14 percent of the U.S.-born black women. The mean birth weight and rates of low birth weight of the infants born to African-born blacks were intermediate between the values in U.S.-born whites and those in U.S.-born blacks. However, when reproductive risk factors were included in the selection of low-risk women, the differences between the infants of U.S.-born whites and the infants of African-born blacks in mean birth weight and rates of both low and very low birth weight were narrowed, whereas the differences between the infants of U.S.-born whites and U.S.-born blacks were unchanged. The greatest change was in very low birth weight; the exclusion of women with a history of fetal loss resulted in nearly identical rates among infants of African-born blacks and those of U.S.-

TABLE 2. DISTRIBUTION OF SELECTED RISK FACTORS IN THE STUDY POPULATION ACCORDING TO THE MOTHER'S RACE AND PLACE OF BIRTH.*

VARIABLE	SUBGROUP OF MOTHERS			RELATIVE RISK (95% CI) IN BLACK MOTHERS†	
	U.S.-BORN WHITES	AFRICAN-BORN BLACKS	U.S.-BORN BLACKS	AFRICAN-BORN	U.S.-BORN
	rate per 100				
Maternal age <20 yr	8.8	1.5	28	0.2 (0.1–0.2)	3.1 (3.0–3.2)
Education <12 yr					
Mother	13	8	36	0.6 (0.5–0.7)	2.9 (2.8–3.0)
Father	11	6	34	0.5 (0.4–0.6)	2.9 (2.9–3.0)
Mother unmarried	14	24	76	1.7 (1.6–1.8)	5.3 (5.2–5.4)
Late prenatal care or none	15	26	36	1.7 (1.6–1.8)	2.3 (2.3–2.4)
Gravidity					
1	34	22	29	0.6 (0.6–0.7)	0.9 (0.8–0.9)
>3	15	31	26	2.0 (1.9–2.1)	1.7 (1.6–1.7)
Prior death					
Fetus‡	24	39	28	1.6 (1.5–1.7)	1.1 (1.1–1.2)
Infant	1.7	3.0	2.9	1.8 (1.5–2.2)	1.7 (1.6–1.9)

*Data on the number of previous pregnancies were obtained for 44,053 U.S.-born white women, 3135 African-born black women, and 43,334 U.S.-born black women. For the other variables shown, there were missing data, as follows: maternal age, 0.01 percent; maternal education, 0.26 percent; paternal education, 16.4 percent; marital status, 0.05 percent; start of prenatal care, 1.38 percent; previous fetal death, 0.07 percent; and previous death of an infant, 0.36 percent.

†Relative risks shown are for the risk of low birth weight in the infants of women in the group shown as compared with the infants of U.S.-born white women. CI denotes confidence interval.

‡This category includes spontaneous and induced abortions, miscarriages, and stillbirths, regardless of the period of gestation.

TABLE 3. REGRESSION MODELS SHOWING THE PREDICTED EFFECTS OF LOW-RISK SOCIODEMOGRAPHIC AND REPRODUCTIVE VARIABLES IN THE MOTHER ON THE BIRTH WEIGHT OF INFANTS IN EACH SUBGROUP DEFINED ACCORDING TO THE MOTHER'S RACE AND PLACE OF BIRTH.*

VARIABLE	SUBGROUP OF MOTHERS		
	U.S.-BORN WHITES (N=44,046)	AFRICAN-BORN BLACKS (N=3135)	U.S.-BORN BLACKS (N=43,322)
	grams		
Birth weight with no protective factors present	3144†	3130†	2942†
Maternal age >19 yr	0	+146†	−25†
Maternal education >11 yr	+128†	−26	+82†
Mother married	+118†	+60‡	+124†
Prenatal care in 1st 3 mo	+60†	−4	+47†
Gravida 2 or 3	+50†	+41	+29†
No prior fetal loss	+19§	+36	+55†

*The values in the table show the increase or decrease in the predicted birth weight in each group, as estimated by arithmetically combining the predicted birth weight with no protective factors present with the sum of the protective factors, each multiplied by 1 if the factor was present or by 0 if it was absent. P values indicate the stability of these point estimates; the greater the standard error of the coefficient, the less the statistical significance.

†P<0.001. ‡P<0.05. §P<0.01.

born whites, eliminating the significant excess of infants with very low birth weight born to African-born blacks.

DISCUSSION

The distribution of birth weights among infants of African-born black women approximated that among infants of U.S.-born white women. The rate of low-birth-weight births for African-born black women was between the rate for U.S.-born white women and that for U.S.-born black women. Adjusting for maternal risk factors in three ways shifted the magnitude of the differences in birth weight but did not alter the basic pattern. Among infants of

TABLE 4. MEAN BIRTH WEIGHTS AND RATES OF LOW BIRTH WEIGHT AMONG INFANTS WITH MOTHERS AT LOW RISK, ACCORDING TO THE MOTHER'S RACE AND PLACE OF BIRTH.

LOW-RISK VARIABLES STUDIED	SUBGROUP OF MOTHERS			RELATIVE RISK (95% CI) IN BLACK MOTHERS*	
	U.S.-BORN WHITES	AFRICAN-BORN BLACKS	U.S.-BORN BLACKS	AFRICAN-BORN	U.S.-BORN
Sociodemographic variables only†					
No. of births	29,012	1577	6181		
Mean birth weight (g)	3,497	3344	3243		
Low birth weight (rate per 100)	3.3	7.0	9.0	2.2 (1.8–2.6)	2.8 (2.5–3.1)
Very low birth weight (rate per 100)	0.6	2.4	1.8	4.3 (3.4–6.2)	3.3 (2.6–4.2)
Reproductive variables added‡					
No. of births	12,361	608	2670		
Mean birth weight (g)	3,551	3454	3299		
Low birth weight (rate per 100)	2.4	3.6	7.5	1.5 (1.0–2.4)	3.0 (2.5–3.5)
Very low birth weight (rate per 100)	0.4	0.5	1.3	1.3 (0.4–4.2)	3.3 (2.2–5.2)

*Relative risks shown are for the risk of low birth weight in the infants of women in the group shown as compared with the infants of U.S.-born white women. CI denotes confidence interval.

†This analysis was limited to women 20 to 39 years of age who began their prenatal care in the first trimester of pregnancy, had at least 12 years of education, and were married to men who also had at least 12 years of education.

‡This analysis was limited as described in the preceding note but also excluded primigravidas and mothers with a history of fetal or infant loss.

African-born black women and those of U.S.-born black women, very low birth weight occurred at a similar frequency. Nevertheless, these data provide some evidence against the theory that there is a genetic basis for the disparity between white and black women born in the United States in the mean birth weights of their infants.

According to most studies, racial differences in birth weight persist independently of numerous social and economic risk factors.[8,9] This has led some investigators to suggest that the differences have a genetic basis.[11-14] Our findings challenge the genetic concept of race as it relates to birth weight. The African-born women in our study were new immigrants from the same region from which the ancestors of most U.S. blacks came, but without the estimated 20 to 30 percent admixture of European genetic material that has occurred since the mid-17th century.[18-21] If genetics played a prominent part in determining black–white differences in birth weight, the infants of the African-born black women should have had lower birth weights than those of the U.S.-born black women. We found the opposite: regardless of socioeconomic status, the infants of black women born in Africa weighed more than the infants of comparable black women born in the United States.

The birth-weight distribution of the infants of African-born black women who delivered in Illinois is consistent with previous reports of the birth weights of infants of foreign-born black women of largely Caribbean origin.[25-28] Studies of groups of women from New York, Boston, and multiple states have had concordant results: black women born outside the United States have heavier infants than those born inside the United States, even after adjustment for cigarette smoking, alcohol intake, and illicit-drug use.

As data inconsistent with the genetic hypothesis of racial differences accumulate, social and psychophysiologic hypotheses are advanced.[5,29-33] A woman's exposure as a young child to the effects of poverty or racial discrimination could adversely affect birth weight in the next generation.[28,34] The high educational level of African-born black women in Illinois indicates that rigorous selection occurs among African immigrants and suggests an overrepresentation of women born into affluent families, an elite subgroup in any developing nation.

Wilcox and Russell, in their extensive work on birth-weight distributions, developed a model that can be applied to the birth-weight curve of any group, partitioning it into an underlying gaussian curve and a "residual" distribution of very-low-birth-weight infants.[35] They proposed that the definition of normal birth weight differs for different groups, on the basis of the underlying distribution in the group under consideration. They attribute the residual births of very-low-birth-weight infants to "disorganized, perhaps pathologic, processes"[35,36] that are presumably environmental in origin.

In our study, the proportions of very-low-birth-weight infants born to African-born black women and to U.S.-born black women were similar. The factors that account for this finding are unclear. As in most published studies, the majority of the risk factors we examined were related to the course of pregnancy. In such a conceptualization, pregnancy is a relatively short-term condition, minimally related to

past life experiences. In an attempt to broaden this concept, we studied how the outcome of prior pregnancy affected the disparity between blacks and whites in rates of very low birth weight. When we controlled for the outcome of prior pregnancy, we found that the rate of very low birth weight among infants of African-born black women more closely resembled that among infants of U.S.-born white women. This observation deserves further investigation.

Our study has important limitations. Vital records contain minimal clinical information. Data on cigarette smoking, weight before pregnancy, and weight gain during pregnancy might, if available, have explained some of our findings. In addition, the group of African-born black women studied, although more than 10 times larger than the group studied previously,[37] was too small to permit stable estimates of very low birth weight in subgroups.

In summary, African-born black women have infants with a greater mean birth weight and a different birth-weight distribution than black women born in the United States.

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Differing Intergenerational Birth Weights among the Descendants of US-born and Foreign-born Whites and African Americans in Illinois

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The authors analyzed Illinois vital records to determine the intergenerational birth weight patterns among the descendants of US-born and foreign-born White and African-American women. Among the descendants of the generation 1 US-born White women (n = 91,061), generation 3 females had a birth weight 65 g more than that of their generation 2 mothers ($p < 0.0001$); generation 3 infants had a 10% lower moderately low birth weight (1,500–2,499 g) rate than did their generation 2 mothers: 5.0% versus 5.5% percent, respectively (relative risk = 0.9, 95% confidence interval: 0.9, 0.9). Among the descendants of generation 1 European-born White women (n = 3,339), generation 3 females had a birth weight 45 g more than that of their generation 2 mothers ($p < 0.0001$). Among the descendants of generation 1 US-born African-American women (n = 31,699), generation 3 females had a birth weight 17 g more than that of their generation 2 mothers ($p < 0.001$). Among the descendants of generation 1 African/Caribbean-born women (n = 104), generation 3 females had a birth weight 57 g less than that of their generation 2 mothers; generation 3 females had a 40% greater moderately low birth weight rate than did their generation 2 mothers: 9.6% percent versus 6.7% percent (relative risk = 1.4, 95% confidence interval: 0.6, 3.6). Maternal age and marital status did not account for the birth weight trends. The authors conclude that the expected intergenerational rise in birth weight does not occur among the direct female descendants of foreign-born African-American women. *Am J Epidemiol* 2002;155:210–16.

Blacks; cohort effect; ethnic groups; infant, low birth weight

In the United States, the mechanisms underlying the disparity in low birth weight (<2,500 g) rates between African-American and White infants are a longstanding epidemiologic enigma and a major public health problem (1, 2). Maternal factors and conditions during pregnancy—age, education, marital status, income, parity, interpregnancy interval, cigarette smoking, and impoverishment—fail to account for the African-American infant's birth weight disadvantage (2–6). However, the limited available data suggest that pregnancy is not an isolated event independent of prior life experiences (7–10). Intergenerational factors are defined as those factors, experiences, and exposures experienced by one generation that relate to the health of the next generation (9). The effect of intergenerational factors on the reproductive outcome of Whites and African Americans is incompletely understood.

We previously found that the birth weight patterns of African-American infants with African-born mothers and White infants with US-born mothers are more closely related to one another than to the birth weights of African-American infants with US-born mothers (11). Consistent with this finding, studies have shown that African-American infants of Caribbean-born mothers also weigh more than African-American infants of US-born mothers independent of maternal risk status during pregnancy (12, 13). These observations suggest that intergenerational factors closely related to lifelong minority status contribute to the African-American women's reproductive disadvantage.

To our knowledge no data have been published on the intergenerational birth weight patterns among the descendants of foreign-born White and African-American women. These populations are uniquely suited to delineate the effect of maternal lifelong minority status on infant birth weight. We therefore undertook an intergenerational birth weight analysis of the direct, female descendants of US-born and foreign-born White and African-American women in Illinois.

MATERIALS AND METHODS

Study population

A detailed description of the Illinois transgenerational data set has been published elsewhere (7). Briefly, the birth certificate data tapes for infants born in 1989–1991 from the Illinois Department of Public Health were linked to those of their mothers who were born in Illinois between 1956 and 1975. There were approximately 328,000 infants in the 1989–1991 cohort with mothers who were also born in

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Illinois. On the basis of each mother's maiden name (first and last) and exact date of birth, we successfully linked 267,604 (79 percent) maternal birth records to infant records. Duplicate matches occurred for 2 percent of infants and were eliminated. After the linkage of maternal and infant birth certificates was complete, all identifying information on the individual mothers and their infants was removed. Thus, the transgenerational file was "sterilized" prior to the initiation of data analyses.

White and African-American mothers in the transgenerational birth file had a slightly better sociodemographic profile than mothers of the 1991 population of Illinois births (7). For example, 9.2 percent of African-American and 2.2 percent of White mothers in the transgenerational file were <18 years of age compared with 12.1 percent of African-American and 2.4 percent of White mothers in the general population, respectively; in addition, 71 percent of African-American and 11 percent of White mothers in the transgenerational file were unmarried compared with 78 percent of African-American and 17 percent of White mothers in the general population, respectively.

Nativity status was empirically defined by maternal grandmother nativity status. Maternal grandmothers were classified as generation 1, mothers (1956–1975 birth cohort) were classified as generation 2, and female infants (1989–1991 birth cohort) were classified as generation 3. The Illinois birth certificates contained a detailed maternal ethnicity variable that included separate codes for "Black," "non-US Black," and "European White." It also contained a maternal nativity variable: It was coded as "Illinois," "other United States," or "remainder of the world." The generational distributions of maternal age and marital status were determined among Whites and African Americans. The birth certificates from the 1956–1975 birth cohort lacked important sociodemographic information such as maternal education and parity.

As a first step toward exploring the possible contribution of maternal lifelong minority status to the racial disparity in pregnancy outcomes, we compared the birth weight distribution curves of generation 2 and generation 3 White and African-American females (i.e., mothers and daughters). Next, we calculated the mean birth weight and the rates of moderately low birth weight (defined as the number of births of infants weighing 1,500–2,499 g) and very low birth weight (defined as the number of births of infants weighing less than 1,500 g) among generation 2 and generation 3 White and African-American females. Finally, we calculated the mean birth weight and moderately low birth weight rates in generation 2 and generation 3 females according to the level of selected sociodemographic characteristics and race.

The 95 percent confidence intervals for the relative risk were calculated by the Taylor series method (14).

RESULTS

Table 1 shows the distribution of young maternal age and unmarried marital status across generations. Among the direct descendants of generation 1 US-born White, European-born White, and US-born African-American women, generation 3 infants had a greater proportion of unmarried mothers than did generation 2 infants. Among the descendants of generation 1 African/Caribbean-born women, generation 3 infants had a greater proportion of both teenaged and unmarried mothers than did generation 2 infants.

TABLE 1. Distribution of selected sociodemographic characteristics in generation 2 and generation 3 females according to generation 1 race and nativity status, Illinois

Generation 1 (maternal grandmothers)	Generation 2 (1956–1975, mothers)		Generation 3 (1989–1991, female infants)	
	Maternal age <20 years (%)	Unmarried marital status (%)	Maternal age <20 years (%)	Unmarried marital status (%)
White				
US born (n = 91,061)	12.2	1.0	9.1*	18.0*
European born (n = 3,339)	5.5	1.0	5.4	9.0*
African American				
US born (n = 31,699)	29.9	14.0	31.9	82.0*
African/Caribbean born (n = 104)	12.5	1.0	34.6*	26.0*

* $p < 0.01$, compares generation 3 with generation 2 according to generation 1 race and nativity status.

Figures 1–4 show race-specific birth weight distribution curves for the direct female descendants (generation 2 and generation 3) of US-born and foreign-born women (generation 1). In both subgroups of Whites, the birth weight distribution curves of generation 3 female infants (compared with their generation 2 mothers) were shifted toward higher birth weights. Among the descendants of US-born African-American women, the birth weight distribution curves of generation 3 female infants were equivalent to that of their generation 2 mothers. Among the descendants of foreign-born African-American women, the distribution curves of generation 3 female infants (compared with their generation 2 mothers) were shifted toward lower birth weights.

Table 2 shows race-specific intergenerational trends in mean birth weight, moderately low birth weight, and very low birth weight rates according to generation 1 (maternal grandmothers) nativity status. Among the descendants of generation 1 US-born White women, generation 3 females had a birth weight 65 g more than that of their generation 2 mothers. Generation 3 infants had a 10 percent lower moderately low birth weight rate and a fourfold greater very low birth weight rate than did their generation 2 mothers. Among the descendants of European-born White generation 1 women, generation 3 females had a birth weight 45 g more than that of their mothers. There were no intergenerational differences in moderately low birth weight rates. There were too few very low birth weight generation 2 infants to calculate meaningful rates.

Among the descendants of generation 1 US-born African-American women, generation 3 females had a birth weight 17g more than that of their generation 2 mothers (table 2). Generation 3 infants had a moderately low birth weight rate equivalent to that of (and a threefold greater very low birth

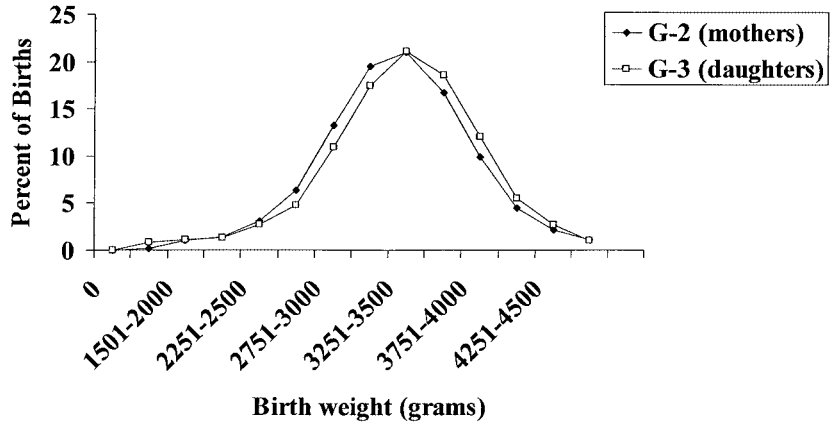

FIGURE 1. Distribution of birth weights among the generation 2 (G-2) and generation 3 (G-3) descendants of generation 1 US-born White women, Illinois, 1956–1975, 1989–1991.

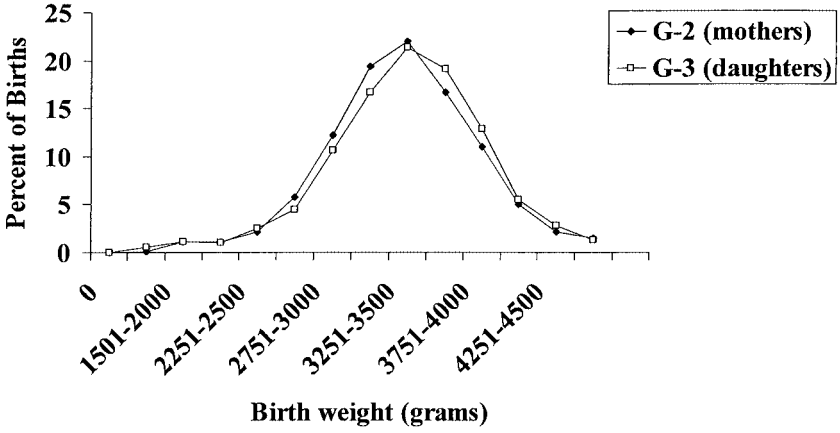

FIGURE 2. Distribution of birth weights among the generation 2 (G-2) and generation 3 (G-3) descendants of generation 1 European-born White women, Illinois, 1956–1975, 1989–1991.

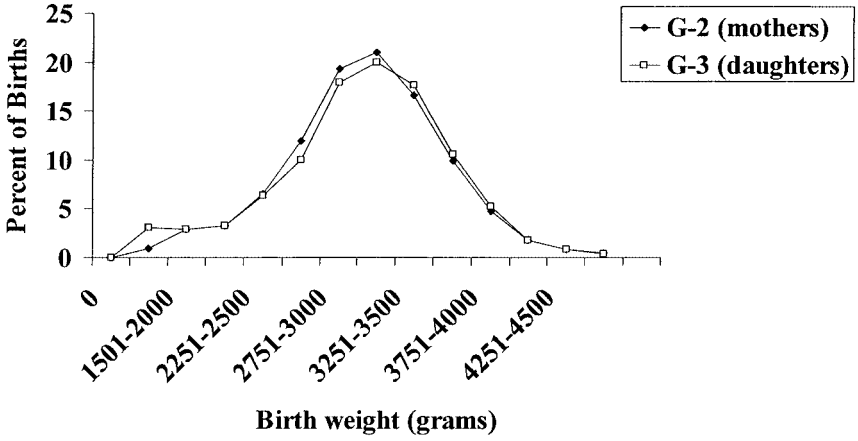

FIGURE 3. Distribution of birth weights among the generation 2 (G-2) and generation 3 (G-3) descendants of generation 1 US-born African-American women, Illinois, 1956–1975, 1989–1991.

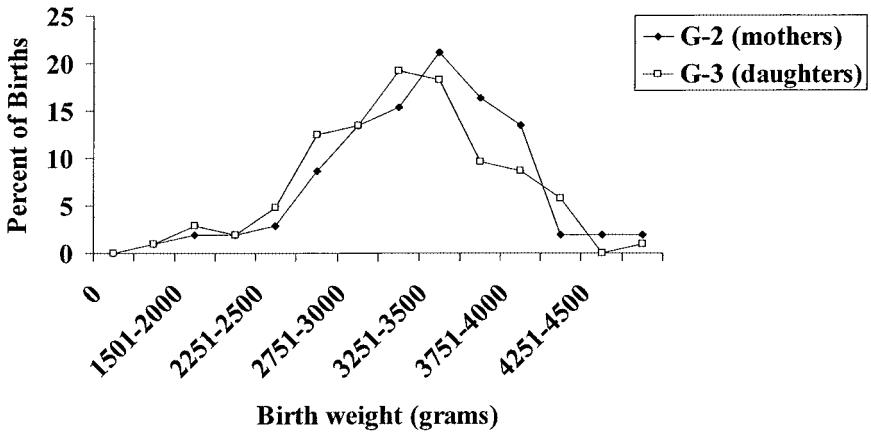

FIGURE 4. Distribution of birth weights among the generation 2 (G-2) and generation 3 (G-3) descendants of generation 1 African/Caribbean-born African-American women, Illinois, 1956–1975, 1989–1991.

weight rate than) their generation 2 mothers. Among the descendants of generation 1 African/Caribbean-born women ($n = 104$), generation 3 females had a birth weight 57 g less than that of their generation 2 mothers (p = not significant). Generation 3 infants had a 40 percent greater moderately low birth weight rate than did their generation 2 mothers (relative risk = 1.4, 95 percent confidence interval: 0.6, 3.6). There were too few generation 2 and generation 3 very low birth weight infants to calculate meaningful rates.

In contrast to Whites, the birth weight of generation 2 African-American infants varied according to generation 1 nativity status (table 2). Generation 2 African-American infants of generation 1 US-born mothers had a 90 percent greater moderately low birth weight rate than did generation 2 African-American infants of generation 1 foreign-born mothers: 12.7 percent versus 6.7 percent (relative risk = 1.9, 95 percent confidence interval: 0.9, 3.8). This differential lessened in the subsequent generation: Generation 3

TABLE 2. Infant birth weight in generation 2 and generation 3 females according to generation 1 race and nativity status, Illinois

Generation 1 (maternal grandmothers)	Generation 2 (1956–1975, mothers)			Generation 3 (1989–1991, daughters)		
	Mean birth weight (g)	1,500–2,499 g (%)	<1,500 g (%)	Mean birth weight (g)	1,500–2,499 g (%)	<1,500 g (%)
White						
US-born ($n = 91,061$)	3,309	5.5	0.2	3,374*	5.0†	0.8‡
European-born ($n = 3,339$)	3,347	4.2	—§	3,392*	4.5¶	0.6
African-American						
US-born ($n = 31,699$)	3,060	12.7	0.9	3,077*	12.5#	3.1**
African/Caribbean-born ($n = 104$)	3,249	6.7	—	3,192	9.6††	—

* $p < 0.001$, compares mean birth weight in generation 3 with that in generation 2 according to generation 1 race and nativity status.

† Relative risk = 0.9 (95% confidence interval: 0.9, 0.9), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

‡ Relative risk = 3.7 (95% confidence interval: 3.2, 4.3), compares percentage of infants (<1,500 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

§ —, undefined, ≤3 infants.

¶ Relative risk = 1.0 (95% confidence interval: 0.8, 1.3), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

Relative risk = 1.0 (95% confidence interval: 1.0, 1.1), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

** Relative risk = 3.3 (95% confidence interval: 2.9, 3.0), compares percentage of infants (<1,500 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

†† Relative risk = 1.4 (95% confidence interval: 0.6, 3.6), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

African-American infants of generation 1 US-born women had only a 30 percent greater moderately low birth weight rate than did generation 3 African Americans of generation 1 foreign-born women: 12.5 percent versus 9.6 percent (relative risk = 1.3, 95 percent confidence interval: 0.7, 2.3).

In both races, the generational trends in birth weight tended to persist among female infants born to nonteenaged and married mothers, respectively (tables 3 and 4).

DISCUSSION

To our knowledge the present study is the first to examine the intergenerational birth weight patterns of the descendants of US-born and foreign-born White and African-American women. We found racial differences in intergenerational birth weight patterns depending on generation 1 nativity status. Among the descendants of generation 1 US-born and European-born White women, the birth weight of generation 3 female infants shifted upward from that of their generation 2 mothers. An intergenerational improvement in birth weight of a substantially smaller magnitude occurs among the descendants of generation 1 US-born African-American women. Most striking, among the direct female descendants of generation 1 foreign-born African-American women, the birth weight of generation 3 infants shifted downward from that of their generation 2 mothers. Intergenerational trends in moderately low birth weight rates tend to parallel that observed in mean birth weight. These findings suggest that maternal lifelong minority status, or something closely related to it, is associated with infant birth weight.

Our data shed new light on the relation between maternal race and infant birth weight in the United States. A 65-g intergenerational increase in mean birth weight and a concurrent 10 percent decrease in moderately low birth weight rates occur among the female descendants of generation 1 US-born White women. This finding is consistent with findings from prior studies showing secular improvements in the mean birth weight on the order of 40–100 g over decades (15, 16). Most striking, only a 17-g intergenerational increase in the mean birth weight and an equivalent moderately low birth weight rate occur among the female descendants of generation 1 US-born African-American women. These disparate racial group trends point to the disquieting speculation that some key measures of African-American women's health in the United States are not improving.

Generation 2 White and African-American women who were themselves born to foreign-born women are uniquely positioned to ascertain the effect of maternal lifelong minority status on infant birth weight. If maternal lifelong minority status did not play a prominent role in determining racial differences in reproductive outcome, the birth weight of generation 3 female African-American infants should follow the same trend observed among generation 3 female White infants and show an upward shift from their generation 2 mothers. We found just the opposite: The mean birth weight of generation 3 female African-American infants shifted downward from that of their generation 2 mothers. Moreover, the 40 percent greater moderately low birth weight rate among generation 3 (compared with generation 2) African-American infants suggests that the deterioration in birth weight is pathologic.

TABLE 3. Birth weight patterns among female infants born to nonteenaged mothers in generation 2 and generation 3 according to generation 1 race and nativity status, Illinois

Generation 1 (maternal grandmothers)	Generation 2 (1956–1975, mothers)*			Generation 3 (1989–1991, daughters)†		
	Mean birth weight (g)	1,500–2,499 g (%)	<1,500 g (%)	Mean birth weight (g)	1,500–2,499 g (%)	<1,500 g (%)
White						
US-born	3,305	5.3	0.2	3,363‡	4.9§	0.8¶
European-born	3,336	4.1	—#	3,385‡	4.1**	0.5‡
African-American						
US-born	3,067	13.5	1.0	3,009‡	15.5††	3.9‡‡
African/Caribbean-born	3,243	6.6	—	3,196	—	—

* Generation 2: White, US-born (n = 79,945) and European-born (n = 3,155); African-American, US-born (n = 22,211) and African/Caribbean-born (n = 91).

† Generation 3: White, US-born (n = 82,768) and European-born (n = 3,160); African-American, US-born (n = 21,587) and African/Caribbean-born (n = 68).

‡ $p < 0.001$, compares mean birth weight in generation 3 with that in generation 2 according to generation 1 race and nativity status.

§ Relative risk = 0.09 (95% confidence interval: 0.9, 0.9), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

¶ Relative risk = 3.6 (95% confidence interval: 3.1, 4.3), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

—, undefined, ≤3 infants.

** Relative risk = 1.0 (95% confidence interval: 0.8, 1.3), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

†† Relative risk = 1.2 (95% confidence interval: 1.1, 1.2), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

‡‡ Relative risk = 3.8 (95% confidence interval: 3.2, 4.4), compares percentage of infants (<1,500 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

TABLE 4. Birth weight patterns among female infants born to married women in generation 2 and generation 3 according to generation 1 race and nativity status, Illinois

Generation 1 (maternal grandmothers)	Generation 2 (1956–1975, mothers)*			Generation 3 (1989–1991, daughters)†		
	Mean birth weight (g)	1,500–2,499 g (%)	<1,500 g (%)	Mean birth weight (g)	1,500–2,499 g (%)	<1,500 g (%)
White						
US-born	3,312	5.5	0.2	3,381‡	4.5§	0.8¶
European-born	3,358	5.0	—#	3,393‡	4.3**	0.5
African-American						
US-born	3,076	12.6	1.1	3,127‡	13.6††	2.3‡‡
African/Caribbean-born	3,250	6.8	—	3,155	12.9§§	—

* Generation 2: White, US-born (n = 90,245) and European-born (n = 3,319); African-American, US-born (n = 27,122) and African/Caribbean-born (n = 103).

† Generation 3: White, US-born (n = 75,036) and European-born (n = 2,986); African-American, US-born (n = 5,782) and African/Caribbean-born (n = 77).

‡ $p < 0.001$, compares mean birth weight in generation 3 with that in generation 2 according to generation 1 race and nativity status.

§ Relative risk = 0.8 (95% confidence interval: 0.8, 0.8), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

¶ Relative risk = 3.1 (95% confidence interval: 2.7, 3.7), compares percentage of infants (<1,500 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

—, undefined, ≤3 infants.

** Relative risk = 0.9 (95% confidence interval: 0.6, 1.2), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

†† Relative risk = 1.1 (95% confidence interval: 1.0, 1.2), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

‡‡ Relative risk = 2.4 (95% confidence interval: 1.9, 2.9), compares percentage of infants (<1,500 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

§§ Relative risk = 2.2 (95% confidence interval: 0.7, 6.8), compares percentage of infants (1,500–2,499 g) in generation 3 with that in generation 2 according to generation 1 race and nativity status.

Our study adds to earlier observations regarding maternal nativity and infant birth weight among African Americans (11–13). As expected, the present study shows that the moderately low birth weight rate of generation 2 African-American infants with US-born mothers exceeds that of generation 2 African-American infants with foreign-born mothers. Moreover, it indicates that the moderately low birth weight rate of generation 3 African-American descendants of generation 1 foreign-born women approaches that of generation 3 African-American descendants of generation 1 US-born women. Given the probable selective migration of healthy generation 1 African-born women (11) and their descendants' worsening birth weight outcomes, we speculate that unidentified aspects of US society are indeed deleterious to the reproductive health of African-American women.

In seeking to understand the mechanisms underlying the birth weight disadvantage of African-American infants with US-born mothers, the dominant concept has been that pregnancy is a relatively acute condition. A corollary is that controlling for maternal age, socioeconomic status, and adequacy of prenatal care usage should largely eliminate racial differences in pregnancy outcome. An extensive literature shows that these pregnancy-related factors and conditions fail to explain birth weight differences between and within the races (2–6, 11–14). The disparate intergenerational birth weight patterns between Whites and African Americans provide evidence that pregnancy, while occurring during a limited time period of a woman's life, should not be considered an isolated event independent of prior life experiences. We encourage researchers to take a woman's prepregnancy (fetal, infant, and childhood) experiences into account when examining racial differences in infant birth weight.

Our study has a number of limitations. First, there was a built-in selection bias in creating the transgenerational birth file. Infants for whom maternal matches were unsuccessful were more likely of low socioeconomic status and thus more prone to low birth weight (7). This would not weaken the main finding that an improvement in intergenerational birth weight does not occur among the descendants of immigrant African-American women. However, it limits that conclusion somewhat in that it is based on observations confined to the less disadvantaged portion of the population. Second, we implicitly assumed that intergenerational improvement in mean birth weight is a good phenomenon. Further research is needed to determine the extent to which it actually lowers mortality and morbidity risk. Third, because of the poor survival of very low birth weight infants in the generation 2 cohort (1956–1975), we were unable to evaluate fully the impact of intergenerational factors on the very low birth weight tail of the birth weight distribution curve. The greater very low birth weight rate among generation 3 (compared with generation 2) infants is an artifact of the Illinois transgenerational birth file. Generation 2 Whites and African Americans in the transgenerational birth file had a very low birth weight rate, approximately one fourth of that of general population births. Since the transgenerational file was defined by generation 3 infants born to generation 2 survivors, this finding is consistent with the high birth

weight-specific mortality rate of very low birth weight generation 2 infants (17). Fourth, the lack of information on maternal educational status in the 1956–1975 birth cohort and the relatively small population of generation 1 African/Caribbean-born women in our data set prevented us from fully evaluating the contribution of generation 1 sociodemographic and nativity status to intergenerational birth weight patterns. Finally, vital records contain minimal clinical information. Maternal weight before pregnancy, weight gain during pregnancy, gestational diabetes, and cesarean-section rates might account for some of our mean birth weight findings.

In summary, the expected intergenerational rise in birth weight does not occur among the female descendants of foreign-born African-American women. It may reflect US-born women's exposure to unidentified intergenerational factors closely linked to minority status (18–20). The identification of such factors will help us attain the Healthy People 2010 goal to eliminate the racial disparity in infant mortality rates (21, 22).

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Supplementary information is available on *Nature's* World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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Mitochondrial genome variation and the origin of modern humans

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The analysis of mitochondrial DNA (mtDNA) has been a potent tool in our understanding of human evolution, owing to characteristics such as high copy number, apparent lack of recombination[1], high substitution rate[2] and maternal mode of inheritance[3]. However, almost all studies of human evolution based on mtDNA sequencing have been confined to the control region, which constitutes less than 7% of the mitochondrial genome. These studies are complicated by the extreme variation in substitution rate between sites, and the consequence of parallel mutations[4] causing difficulties in the estimation of genetic distance and making phylogenetic inferences questionable[5]. Most comprehensive studies of the human mitochondrial molecule have been carried out through restriction-fragment length polymorphism analysis[6], providing data that are ill suited to estimations of mutation rate and therefore the timing of evolutionary events. Here, to improve the information obtained from the mitochondrial molecule for studies of human evolution, we describe the global mtDNA diversity in humans based on analyses of the complete mtDNA sequence of 53 humans of diverse origins. Our mtDNA data, in comparison with those of a parallel study of the Xq13.3 region[7] in the same individuals, provide a concurrent view on human evolution with respect to the age of modern humans.

The molecular clock hypothesis postulates that DNA sequence evolution is roughly constant over time in all evolutionary lineages. We used a test[8] that compares the log likelihoods of trees reconstructed with and without the molecular clock assumption to examine the supposition that the mtDNA lineages evolve at 'clock-like' rates. The human mtDNA sequences, excluding the D-loop, have evolved at roughly constant rates ($P = 0.094$), and a relative rates test[9], using a gorilla sequence as an outgroup, demonstrates that there is also no significant difference between the evolutionary rate of human and chimpanzee mtDNAs ($P = 0.123$), excluding the D-loop. In contrast, the D-loop has not evolved at a constant rate across all human lineages ($P < 0.001$), and is consequently less suitable for dating evolutionary events. Therefore, unless specifically mentioned, we have excluded the D-loop from the analyses that follow.

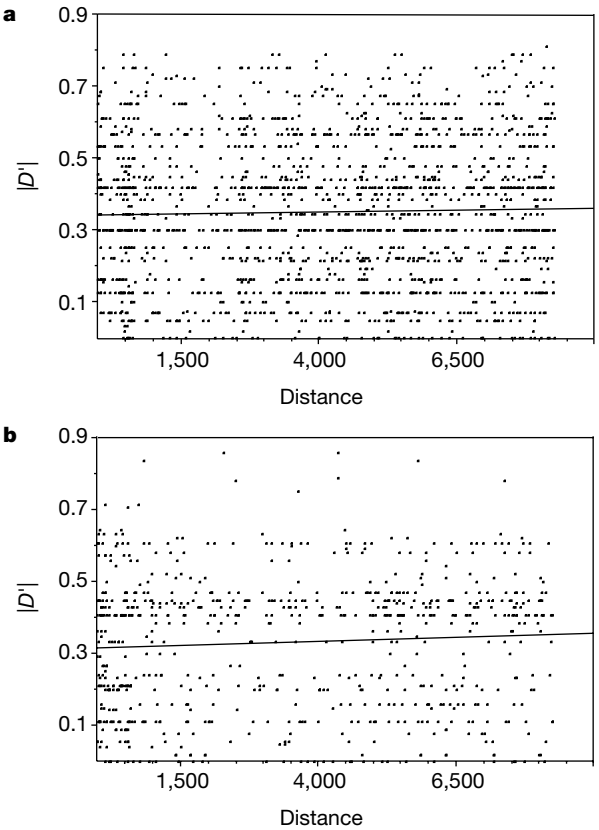


Figure 1 The relationship between linkage disequilibrium, measured by $|D'|$ versus distance between nucleotide sites for all 53 complete human mtDNA genomes. Values of ± 1.0 have been removed. **a**, Individuals of African descent ($n = 1{,}719$ comparisons), $R^2 = 0.001$; **b**, only non-African individuals ($n = 741$ comparisons), $R^2 = 0.005$.

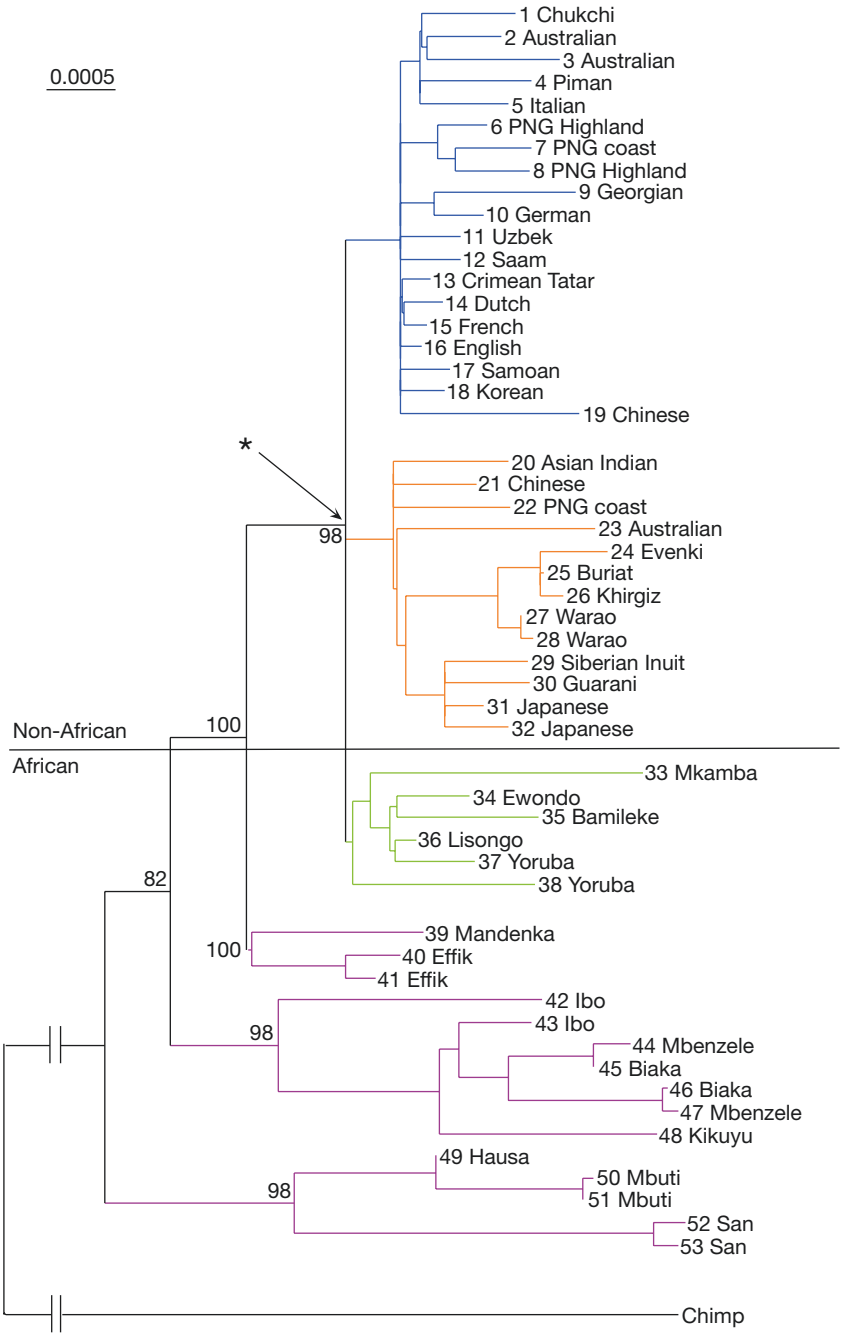


Figure 2 Neighbour-joining phylogram based on complete mtDNA genome sequences (excluding the D-loop). Data was constructed using PAUP*4.0 Beta (Sinauer Associates) and bootstrapped with 1,000 replicates (bootstrap values shown on nodes). The population origin of the individual is given at the twigs. Branches have been colour coded as in Fig. 4. Individuals of African descent are found below the dashed line and non-Africans above. The node marked with an asterisk refers to the MRCA of the youngest clade containing both African and non-African individuals.

Table 1 Summary of statistical parameters for the mtDNA

	Data set	Length	*n*	*S*	MPSD	π
Total	All humans	16,553	53	657	61.1	3.7×10^{-3}
	Non-Africans	16,555	32	358	38.5	2.3×10^{-3}
	Africans	16,556	21	367	76.7	4.6×10^{-3}
D-Loop	All humans	1,118	53	141	17.2	1.5×10^{-2}
	Non-Africans	1,118	32	103	12.8	1.1×10^{-2}
	Africans	1,121	21	77	19.7	1.8×10^{-2}
Rest	All humans	15,435	53	516	43.9	2.8×10^{-3}
	Non-Africans	15,437	32	255	25.7	1.7×10^{-3}
	Africans	15,448	21	290	57.0	3.7×10^{-3}

mtDNA data are given as entire sequences, or separated into D-loop and rest (all but the D-loop), and further separated into groups of African and non-African individuals. Length, aligned sequence length excluding gaps; *n*, number of sequences; *S*, number of segregating sites; MPSD, mean pairwise sequence difference; and π, genetic diversity.

From the mean genetic distance between all the humans and the one chimpanzee sequence (0.17 substitutions per site) and the assumption, based on palaeontological[10] and genetic[11] evidence, of a divergence time between humans and chimpanzees of 5 Myr, the mutation rate (μ) for the mitochondrial molecule, excluding the D-loop, is estimated to be 1.70×10^{-8} substitutions per site per year.

On the basis of the correlation between linkage disequilibrium and distance between sites, it has been claimed that mtDNA sequences show signs of recombination[12,13]. These analyses can be criticized for the methodology used[14], particularly for the use of a linkage disequilibrium measure (Hill and Robertson measure, r^2) that doesn't take allele frequency into account. We examined linkage

disequilibrium among all informative sites in our set of complete mtDNA genomes (including the D-loop) using a standard estimate (D') that allows all variable positions to be examined[15]. For this analysis, we studied the sequences of Africans and non-Africans separately. There is no correlation between D' and nucleotide distance between sites in the 53 sequences (African correlation coefficient, $R^2 = 1 \times 10^{-3}$; non-African $R^2 = 5 \times 10^{-3}$) (Fig. 1). We also analysed the association between r^2 and distance, and again there is no evidence of a correlation ($R^2 = 2.23 \times 10^{-6}$, and 1×10^{-3}, respectively; data not shown). Thus, it does not appear to be necessary to consider recombination as contributing to the evolution of mtDNA.

The two main hypotheses for the evolution of modern humans agree that *Homo erectus* spread from Africa around 2 Myr ago. The 'recent African origin' hypothesis[16,17] states that anatomically modern humans originated in Africa 100,000–200,000 years ago and subsequently spread to the rest of the world, replacing archaic human forms with little or no genetic mixing. The alternative, 'multi-regional' hypothesis proposes that the transformation to anatomically modern humans occurred in different parts of the world, and supports this with fossil evidence of cultural and morphological continuity between archaic and modern humans outside Africa[18]. There are, of course, variants of these two basic hypotheses that introduce additional assumptions, such as gene flow, that make the hypotheses increasingly difficult to test.

Support for a recent African origin of modern humans has been provided by a number of mtDNA studies[16,17,19,20]; however, these results have been troubled by the lack of statistical support for tree topology, especially the deep African branches[21,22]. Lacking sufficiently strong empirical data, it is impossible to confidently place the root of modern human mtDNA lineages in sub-Saharan Africa. The neighbour-joining[23] (NJ) tree constructed from our mtDNA sequences has a strongly supported basal branching pattern (Fig. 2). The three deepest branches lead exclusively to sub-Saharan mtDNAs, with the fourth branch containing both Africans and non-Africans. The deepest, statistically supported branch (NJ bootstrap = 100) provides compelling evidence of a human mtDNA origin in Africa.

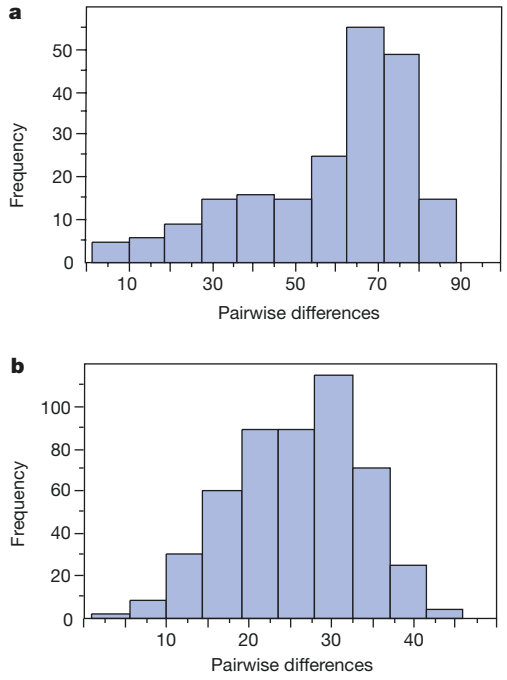


Figure 3 Mismatch distributions of pairwise nucleotide differences between mtDNA genomes (excluding the D-loop). **a**, African; **b**, non-African.

The amount of mtDNA sequence diversity (π) among Africans (3.7×10^{-3} nucleotide differences per site) is more than twice that among non-Africans (1.7×10^{-3}) (Table 1), corroborating earlier studies of the D-loop[16] and nuclear loci[24]. Also notable is the contrast between the deep branches of African mtDNAs and the 'star-like' phylogeny of non-African mtDNAs (Fig. 2). This high African diversity might result from either a considerably larger effective population size or a significantly longer genetic history. The 'star-like' phylogeny of the non-African sequences suggests a

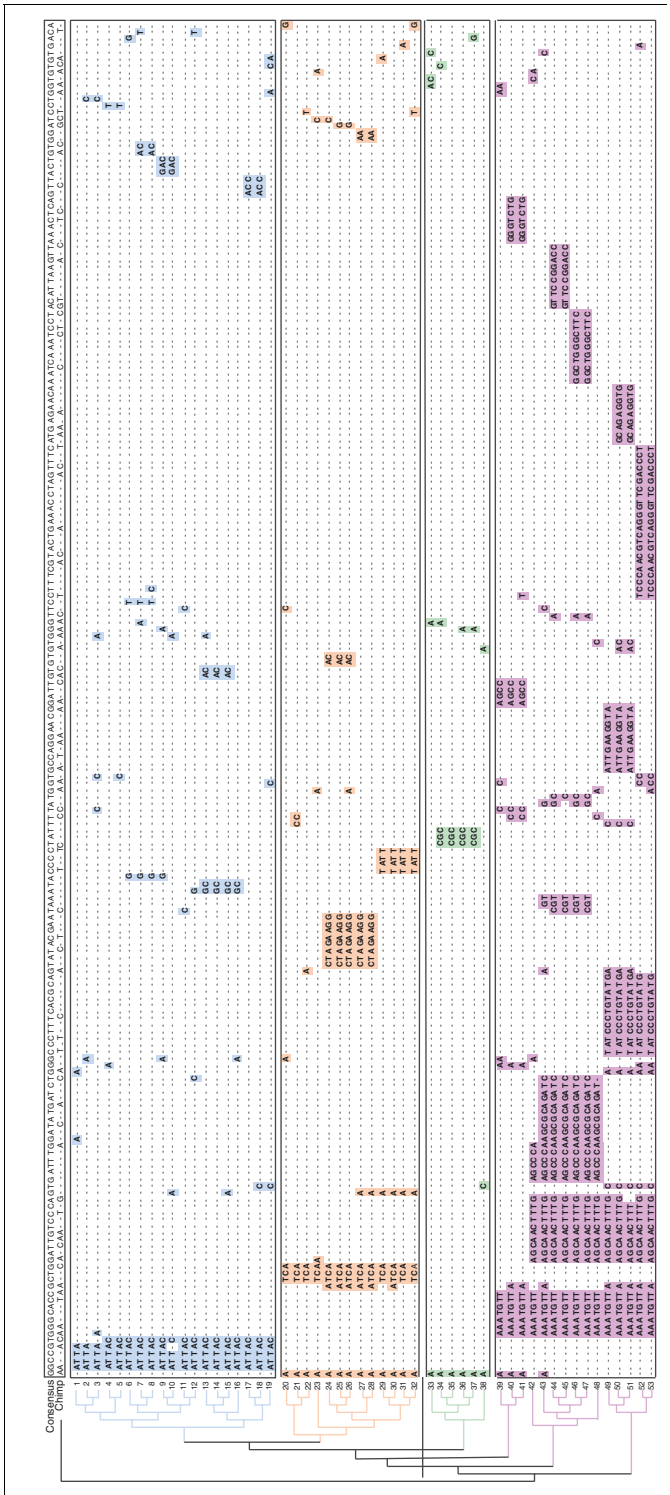
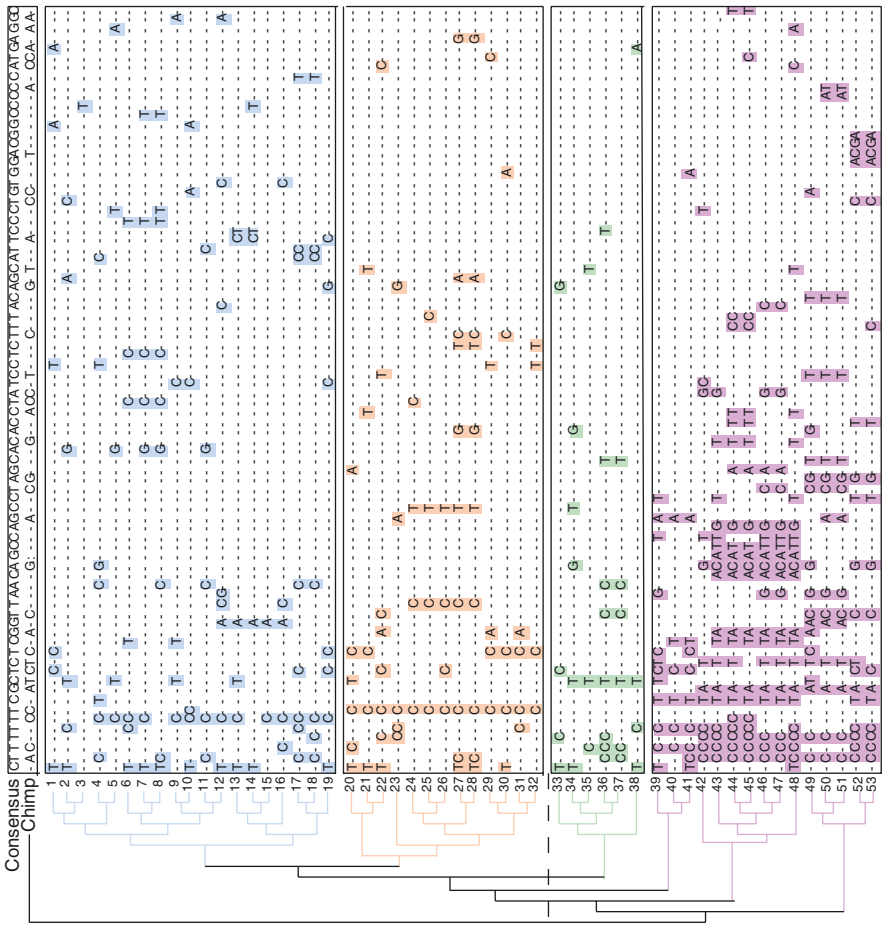


Figure 4 Data matrices showing all informative nucleotide positions, in decreasing order of frequency. Left, the whole mtDNA genome, excluding the D-loop. Right, the D-loop. The

population bottleneck, potentially associated with the colonization of Eurasia from Africa. The date of this exodus from Africa can be estimated if the departing group subsequently experienced a population expansion. The mtDNA mismatch distributions for Africans and non-Africans indicate a marked difference in population history for the two groups[25] (Fig. 3). Mitochondrial DNAs from individuals of African origin show a ragged distribution consistent with constant population size, whereas the bell-shaped distribution of the non-African comparisons clearly indicates a recent population expansion. The assumption of constant population size can be verified by tests of selective neutrality that examine the correlation between the mean pairwise sequence difference (MPSD) and the number of segregating sites (S)[26,27]. In the African group, we cannot reject this assumption (Fu and Li's $D = -1.17$ (ref. 26); Tajima's $D = -1.22$ (ref. 27)), consistent with the premise that the population has been of roughly constant size . However, it can be rejected in the non-African group ($D = -4.02$; $D = -2.28$), indicating that this group has experienced a period of population growth[25]. The



trees on the left are cladograms with the same topology and numbering of individuals as the tree in Fig. 2. Individuals of African descent are found below the dashed line and non-Africans above. The four major groups of sequences have been colour coded as in Fig. 2. Blocks denote groups of nucleotides that are identical in several sequences.

time when the expansion began was estimated ($\tau = 20.23$) to be about 1,925 generations ago[28]. Assuming a generation time of 20 yr this equates to 38,500 yr BP, a date that coincides with the onset of a period of cultural change about 35,000–40,000 years ago[29]. This involves, for example, the first appearance of regional cultural variation and the acceleration of artefactual change.

The age of the most recent common ancestor (MRCA) for mtDNA, on the basis of the maximum distance between two humans (5.82×10^{-3} substitutions per site between the Africans Mkamba and San), is estimated to be $171{,}500 \pm 50{,}000$ yr BP. We can also estimate the age of the MRCA for the youngest clade that contains both African and non-African sequences (Fig. 2, asterisk) from the mean distance of all members of that clade to their common node (8.85×10^{-4} substitutions per site) as $52{,}000 \pm 27{,}500$ yr BP. Because genetic divergence is expected to precede the divergence of populations, this date can be considered as the lower bound for an exodus from Africa.

Notably, a group of six African sequences (Fig. 4a, sequences 33–38) are genetically distant to those of other Africans, but share a common ancestor with non-Africans. These lineages represent descendants of a population that evidently gave rise to all the non-African lineages. Whether the ancestors of these six extant lineages originally came from a specific geographic region is not possible to determine, but we note that these sequences are from five populations that are now geographically unrelated.

Our study of the entire mitochondrial genome has some significant distinctions from previous studies of the D-loop. Most notably, the sequences outside of the D-loop evolve in a roughly 'clock-like' manner, enabling a more accurate measure of mutation rate, and therefore improved estimates of times to evolutionary events. Also of importance is the strong statistical support for the tree topology that has been lacking in earlier investigations. The difference between the D-loop and the rest of the molecule is visually evident in the contrast between the jumbled arrangement of polymorphic sites in the D-loop and the clear haplotypes defined by the sites in the rest of the molecule (Fig. 4).

The use of largely the same individuals in the this study, as in that of the nuclear Xq13.3 (ref. 7) region, provides a unique opportunity to compare the information gained from the two genetic systems. Because the X chromosome has an effective population size three times that of mitochondria, the MRCA of an X-chromosomal locus is expected to be three times higher. Thus, the age of the MRCA of Xq13.3 is in agreement with the mtDNA data (mtDNA: 171,500; Xq13.3: 479,000 yr BP). The results from Xq13.3 also concur with the mtDNA data with respect to the greater genetic diversity found among African individuals relative to non-Africans, but Xq13.3 shows a considerably lower difference in diversity between the two groups (mtDNA: African, 3.9×10^{-3}, non-African, 1.7×10^{-3}; Xq13.3 : 3.5×10^{-4}, 3.05×10^{-4}). Owing to its lower substitution rate, only 33 segregating sites were present in 69 sequences of 10.2 kilobases (kb) at Xq13.3, as compared with the 657 segregating sites in the mitochondrial dataset. Comparisons of the mtDNA and Xq13.3 sequences carried by specific individuals show little correlation between the two loci, as expected from the different modes of inheritance. For example, the two Warao indians showing the highest similarity in mtDNA have, in fact, two of the most divergent sequences studied at the Xq13.3 locus. Others, such as the Saami and Mandenka sequences, are closely related at Xq13.3 but have relatively high mitochondrial divergence.

Our results indicate that the field of mitochondrial population genomics will provide a rich source of genetic information for evolutionary studies. Nevertheless, mtDNA is only one locus and only reflects the genetic history of females. For a balanced view, a combination of genetic systems is required. With the human genome project reaching fruition, the ease by which such data may be generated will increase, providing us with an evermore detailed understanding of our genetic history. □

Methods

Sampling strategy and mtDNA sequences

To assess the global genetic diversity in humans, while analysing a restricted number of samples, we studied 53 individuals representing 14 of the major linguistic phyla. This sampling strategy attempts to avoid the bias inherent in selecting individuals on the basis of current world demographics, such as current population size or geographic location[5]. To provide an opportunity for comparison, we selected, where possible, the same individuals as those used by a previous study[7] for the analysis of the Xq13.3 region. All the complete mtDNA sequences are unique and vary in length from 16,558 to 16,576 base pairs (bp). From nearly 900 kb sequenced, 5 heteroplasmic sites were confidently identified. We identified a total of 657 segregating sites (141 in the D-loop; 516 outside) among these 53 individuals, of which 283 (80 in the D-loop; 203 outside) showed the same polymorphism in at least 2 individuals (Fig. 4). The pairwise sequence distances between mtDNAs, corrected for multiple substitutions[4], vary from 6.0×10^{-5} substitutions per site between two South American indians (Warao) to 6.8×10^{-3} substitutions per site between two Africans (Mbenzele pygmy and San). The average distance between mtDNA genomes is 3.8×10^{-3} substitutions per site.

PCR primers and sequencing

The primers we used for polymerase chain reaction (PCR) amplification have been described[30]. Sequencing was performed on the PCR products directly using BigDye (Applied Biosystems) chemistry. Separation of sequencing ladders was performed on the ABI 377 instrument for automated fragment analysis. We sequenced both forward and reverse strands. Sequence analysis was performed using Sequencing Analysis 3.3 (Applied Biosystems), and sequence alignment was made with Sequencher 3.1.1 (Gene Codes).

Accession numbers

The chimpanzee sequence (GenBank accession no. D38113 and the gorilla sequence (accession no. D38114) that we used as outgroups were obtained from a public mitochondrial sequence database (MITOMAP: http://www.gen.emory.edu/ mitomap.html).

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ties of the compounds manometrically (*5*). In the other, the material is applied to the paper along 8 cm of the base line rather than as a spot and, after resolution, areas 8 × 5 cm containing the various compounds are cut from the paper and rolled in shell vials. Ten anesthetized houseflies are then introduced into each vial, and the toxicity of the compounds is characterized by rate of knockdown and 24-hr mortality.

The paper chromatographic method is useful in studying the metabolism of phosphorus insecticides in plants, mammals, and insects. With it, for example, we have been able to demonstrate the conversion of parathion and its methyl analog to the corresponding phosphates by an enzyme system found in *Periplaneta americana* (L.) (*2*). Further studies are in progress. The method has also been of value in studying the action of heat on purified parathion and methyl parathion and in isolating the compounds formed and in studying their biological properties (*1*).

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A Production of Amino Acids Under Possible Primitive Earth Conditions

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The idea that the organic compounds that serve as the basis of life were formed when the earth had an atmosphere of methane, ammonia, water, and hydrogen instead of carbon dioxide, nitrogen, oxygen, and water was suggested by Oparin (*1*) and has been given emphasis recently by Urey (*2*) and Bernal (*3*).

In order to test this hypothesis, an apparatus was built to circulate CH_4, NH_3, H_2O, and H_2 past an electric discharge. The resulting mixture has been tested for amino acids by paper chromatography. Electrical discharge was used to form free radicals instead of ultraviolet light, because quartz absorbs wavelengths short enough to cause photo-dissociation of the gases. Electrical discharge may have played a significant role in the formation of compounds in the primitive atmosphere.

The apparatus used is shown in Fig. 1. Water is boiled in the flask, mixes with the gases in the 5-l flask, circulates past the electrodes, condenses and empties back into the boiling flask. The U-tube prevents circulation in the opposite direction. The acids and amino acids formed in the discharge, not being volatile, accumulate in the water phase. The circulation of the gases is quite slow, but this seems to be an asset, because production was less in a different apparatus with an aspirator arrangement to promote circulation. The discharge, a small corona, was provided by an induction coil designed for detection of leaks in vacuum apparatus.

The experimental procedure was to seal off the opening in the boiling flask after adding 200 ml of water, evacuate the air, add 10 cm pressure of H_2, 20 cm of CH_4, and 20 cm of NH_3. The water in the flask was boiled, and the discharge was run continuously for a week.

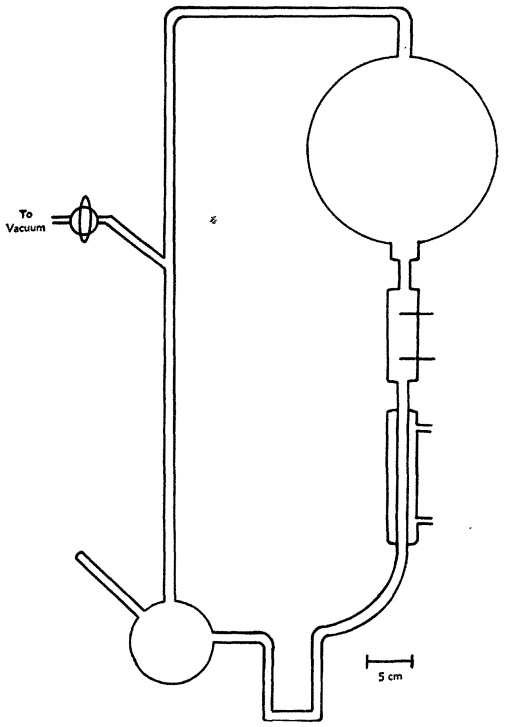

FIG. 1.

During the run the water in the flask became noticeably pink after the first day, and by the end of the week the solution was deep red and turbid. Most of the turbidity was due to colloidal silica from the glass. The red color is due to organic compounds adsorbed on the silica. Also present are yellow organic compounds, of which only a small fraction can be extracted with ether, and which form a continuous streak tapering off at the bottom on a one-dimensional chromatogram run in butanol-acetic acid. These substances are being investigated further.

At the end of the run the solution in the boiling flask was removed and 1 ml of saturated $HgCl_2$ was added to prevent the growth of living organisms. The ampholytes were separated from the rest of the constituents by adding $Ba(OH)_2$ and evaporating *in vacuo* to remove amines, adding H_2SO_4 and evaporat-

[1] National Science Foundation Fellow, 1952–53.

[2] Thanks are due Harold C. Urey for many helpful suggestions and guidance in the course of this investigation.

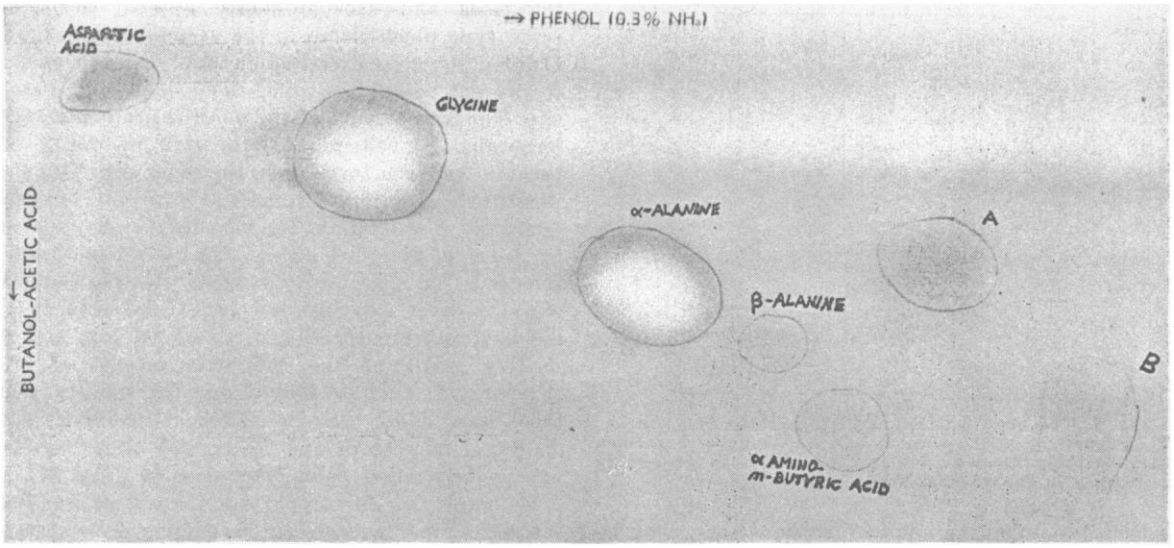

FIG. 2.

ing to remove the acids, neutralizing with $Ba(OH)_2$, filtering and concentrating *in vacuo*.

The amino acids are not due to living organisms because their growth would be prevented by the boiling water during the run, and by the $HgCl_2$, $Ba(OH)_2$, H_2SO_4 during the analysis.

In Fig. 2 is shown a paper chromatogram run in *n*-butanol-acetic acid-water mixture followed by water-saturated phenol, and spraying with ninhydrin. Identification of an amino acid was made when the R_f value (the ratio of the distance traveled by the amino acid to the distance traveled by the solvent front), the shape, and the color of the spot were the same on a known, unknown, and mixture of the known and unknown; and when consistent results were obtained with chromatograms using phenol and 77% ethanol.

On this basis glycine, α-alanine and β-alanine are identified. The identification of the aspartic acid and α-amino-*n*-butyric acid is less certain because the spots are quite weak. The spots marked A and B are unidentified as yet, but may be beta and gamma amino acids. These are the main amino acids present, and others are undoubtedly present but in smaller amounts. It is estimated that the total yield of amino acids was in the milligram range.

In this apparatus an attempt was made to duplicate a primitive atmosphere of the earth, and not to obtain the optimum conditions for the formation of amino acids. Although in this case the total yield was small for the energy expended, it is possible that, with more efficient apparatus (such as mixing of the free radicals in a flow system, use of higher hydrocarbons from natural gas or petroleum, carbon dioxide, etc., and optimum ratios of gases), this type of process would be a way of commercially producing amino acids.

A more complete analysis of the amino acids and other products of the discharge is now being performed and will be reported in detail shortly.

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2. UREY, H. C. *Proc. Natl. Acad. Sci. U. S.*, **38**, 351 (1952); *The Planets*. New Haven: Yale Univ. Press. Chap. 4 (1952).
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Manuscript received February 13, 1953.

A Vacuum Microsublimation Apparatus

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The analytical biochemist is frequently confronted with the task of isolating microquantities of substances in a chemically pure state from small quantities of tissues or biological fluids. Kofler (*1*) edited a book covering the use of microsublimation, melting point, eutectics, etc., in identifying microquantities of organic material. The advantages of sublimation over other methods of purification have been discussed by Hubacher (*2*). Many types of vacuum sublimation apparatus have been described (*1–3*). The equipment described here is inexpensive and can be assembled readily by any laboratory worker with a modicum of glassblowing skill.

To a thick-walled, round-bottom, Pyrex test tube, 30 × 200 mm, is attached a glass side arm about one in. from the bottom. Using a suspension of very fine emery in glycerin or fine valve-grinding compound, the open end of the test tube is ground against the aluminum block of a Fisher-Johns melting point apparatus (Fisher Scientific Co., St. Louis, Mo.) until it makes a vacuum-tight seal when dry. This is the vacuum hood. Microbeakers are prepared from flat-

[1] The author is indebted to Robert Puckett, of this laboratory, for technical assistance in preparing this apparatus.

The Miller Volcanic Spark Discharge Experiment

Adam P. Johnson,[1] H. James Cleaves,[2] Jason P. Dworkin,[3] Daniel P. Glavin,[3] Antonio Lazcano,[4] Jeffrey L. Bada[5]*

In 1953, Miller (*1*) published a short paper describing the spark discharge synthesis of amino acids from a reducing gas mixture thought to represent the atmosphere of the early Earth. This experiment showed that the basic molecules of life could be synthesized from simple molecules, suggesting that Darwin's "warm little pond" was a feasible scenario.

After Miller's death on 20 May 2007, we found several boxes containing vials of dried residues. Notebooks (*2*) indicated that the vials came from his 1953–54 University of Chicago experiments that used three different configurations (*3*, *4*). One was the original apparatus used in (*1*). Another incorporated an aspirating nozzle attached to the water-containing flask, injecting a jet of steam and gas into the spark. The third incorporated the aspirator device but used a silent discharge instead of electrodes. Although Miller repeated his experiment in 1972 with use of the original architecture (*5*), the others were never tested again.

We were interested in the second apparatus because it possibly simulates the spark discharge synthesis by lightning in a steam-rich volcanic eruption (*6*) (Fig. 1A). Miller identified five different amino acids, plus several unknowns, in the extracts from this apparatus (*3*). Product yields appeared somewhat higher than those in the classical configuration, although Miller never confirmed this. We reanalyzed 11 vials in order to characterize the diversity of products synthesized in this apparatus.

The residues in the vials were resuspended in 1-ml aliquots of doubly distilled deionized water and characterized by high-performance liquid chromatography and liquid chromatography–time of flight mass spectrometry that allows for identification at the sub-picomolar ($<10^{-12}$ M) level (*2*). We identified 22 amino acids and five amines in the volcanic experiment (Fig. 1C), several of which had not been previously identified in Miller's experiments. Vials from the other two experiments were also reanalyzed and found to have a lower diversity of amino acids (table S1). The yield of amino acids synthesized in the volcanic experiment is comparable to, and in some cases exceeds, those found in the experiments Miller conducted (*1*, *3*, *5*). Hydroxylated compounds were preferentially synthesized in the volcanic experiment. Steam injected into the spark may have generated OH radicals that reacted with either the amino acid precursors or the amino acids themselves (*7*).

Geoscientists today doubt that the primitive atmosphere had the highly reducing composition Miller used. However, the volcanic apparatus experiment suggests that, even if the overall atmosphere was not reducing, localized prebiotic synthesis could have been effective. Reduced gases and lightning associated with volcanic eruptions in hot spots or island arc–type systems could have been prevalent on the early Earth before extensive continents formed (*8*). In these volcanic plumes, HCN, aldehydes, and ketones may have been produced, which, after washing out of the atmosphere, could have become involved in the synthesis of organic molecules (*3*, *4*, *8*). Amino acids formed in volcanic island systems could have accumulated in tidal areas, where they could be polymerized by carbonyl sulfide, a simple volcanic gas that has been shown to form peptides under mild conditions (*9*).

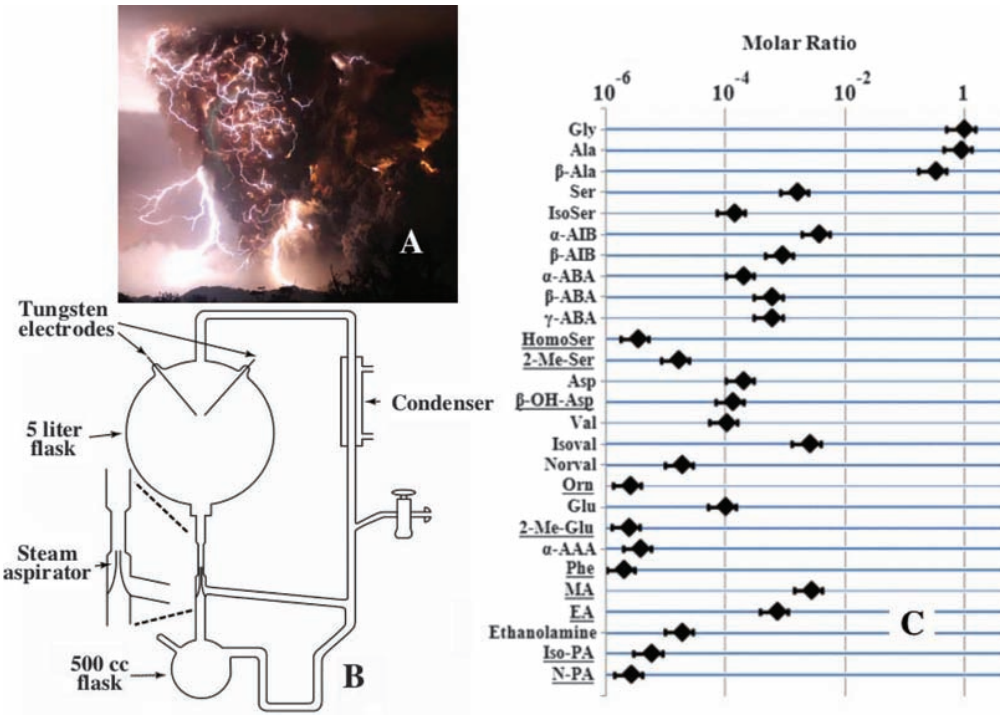

Fig. 1. (**A**) Lightning associated with the 3 May 2008 eruption of the Chaiten volcano, Chile. [Photo credit: Carlos Gutierrez/UPI/Landov] (**B**) The volcanic spark discharge apparatus used by Miller (*3*). Gas quantities added were 200 torr of CH_4, 200 torr of NH_3, and 100 torr of H_2 [these would have dissolved in the water according to their solubilities (*2*)]. Water was added to the 500-cm^3 (cc) flask and boiled, and the apparatus sparked with a Tesla coil for 1 week; (**C**) Moles (relative to glycine = 1) of the various amino acids detected in the volcanic apparatus vials [see (*2*) and table S1 for abbreviations]. Amino acids underlined have not been previously reported in spark discharge experiments. Values for amines are minimum values because of loss due to their volatility during workup.

References and Notes

1. S. L. Miller, *Science* **117**, 528 (1953).
2. Analytical details and additional data are available as supporting material on *Science* Online.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/322/5900/404/DC1
Materials and Methods
Figs. S1 and S2
Table S1
References and Notes

9 June 2008; accepted 8 August 2008
10.1126/science.1161527

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*To whom correspondence should be addressed. E-mail: jbada@ucsd.edu

Prof or LA interview questions

(you will answer 10 Qs; chosen randomly; audiotaped; can bring papers but no notes)

1. What was the official lab assignment, what are you doing this semester?
2. Pretend I am your mom/dad. Explain to me your research in lay-terms without diluting your purpose/methods/finding.
3. What makes your project same vs different from previous publications?
4. What research paper(s) is the most influential for your project (show me)?
5. Provide brief explanations about the significance and/or importance of background literature you have used?
6. Pull out a hard copy of one of the research papers you've read for your research project and explain to me this {title/figure/abstract/methods}.
7. Pull out a hard copy of the {Ulagaraj-crickets, Fabricius-coral, Lewis-firefly, Manser-meerkats} paper and explain this {title/figure/abstract/methods}.
8. What is the difference between a hypothesis and a prediction? Examples?
9. What are the scientific and common names of the organisms you study?
10. What is your control vs. treatment(s) in your experiment, and variables?
11. Define, provide examples, what is a positive control vs. negative control?
12. How are you using: randomization, replication, sample size; in your study?
13. What are your hypotheses for your entire research project? What are the associated predictions for each hypothesis?
14. What specific data sets will you be collecting in your studies of either animal-type? (What did they do in the publication you are replicating?)
15. Describe how you will collect data for homologous human behavior study?
16. Identify relevance & purpose of finding homologous behaviors in humans?
17. How do you plan to analyze your data?
18. Can you provide information on the gene that is associated with the communication behavior? (Give specifics: located? base pairs? function?)
19. How does your gene correlate with your behavior, what pathologies would be predicted to occur if your gene gets knocked-out?
20. Imagine you are in an interview for your dream job, or for your dream med/vet/grad school, in 5 sentences, explain the research project you led this semester and impress me so much you can feel confident "you got it".

Homework (at the end, you will then do one of these; <20 minutes):

A. Here is a set of data, calculate the average and generate error bars. How do we do a statistical test to determine if the two sets are different? If you run a statistical test and the p-value is calculated to be 0.04, and your significance level is 0.05, interpret the meaning of your p-value obtained from the statistical test. Does it prove significance? Why?

B. Draw me a labeled graph that accurately represents your predicted data (what you are predicting to be the results of your experiment).

C. Using HTML expertise, code a {X} page that would look like one expected in a formal manuscript and abides by the rules of Instructions to Authors.

D. Take your smartphone and to film <10 seconds of the behavior of that {fish, student, squirrel} and then create for me an HTML page with it as a extraordinarily professional polished scientific Figure that includes a self-starting looped video figure (like seen in a Harry Potter newspaper).

Prof or LA interview questions

(you will answer 10 Qs; chosen randomly; audiotaped; can bring papers but no notes)

1. What was the official lab assignment, what are you doing this semester?
2. Pretend I am your mom/dad. Explain to me your research in lay-terms without diluting your purpose/methods/finding.
3. What makes your project same vs different from previous publications?
4. What research paper(s) is the most influential for your project (show me)?
5. Provide brief explanations about the significance and/or importance of background literature you have used?
6. Pull out a hard copy of one of the research papers you've read for your research project and explain to me this {title/figure/abstract/methods}.
7. Pull out a hard copy of the {Ulagaraj-crickets, Fabricius-coral, Lewis-firefly, Manser-meerkats} paper and explain this {title/figure/abstract/methods}.
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9. What are the scientific and common names of the organisms you study?
10. What is your control vs. treatment(s) in your experiment, and variables?
11. Define, provide examples, what is a positive control vs. negative control?
12. How are you using: randomization, replication, sample size; in your study?
13. What are your hypotheses for your entire research project? What are the associated predictions for each hypothesis?
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15. Describe how you will collect data for homologous human behavior study?
16. Identify relevance & purpose of finding homologous behaviors in humans?
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Homework (at the end, you will then do one of these; <20 minutes):

A. Here is a set of data, calculate the average and generate error bars. How do we do a statistical test to determine if the two sets are different? If you run a statistical test and the p-value is calculated to be 0.04, and your significance level is 0.05, interpret the meaning of your p-value obtained from the statistical test. Does it prove significance? Why?

B. Draw me a labeled graph that accurately represents your predicted data (what you are predicting to be the results of your experiment).

C. Using HTML expertise, code a {X} page that would look like one expected in a formal manuscript and abides by the rules of Instructions to Authors.

D. Take your smartphone and to film <10 seconds of the behavior of that {fish, student, squirrel} and then create for me an HTML page with it as a extraordinarily professional polished scientific Figure that includes a self-starting looped video figure (like seen in a Harry Potter newspaper).

Film Evaluation[1] (LBC Universal Communication Rubric)

1. Does this film have an appropriate and interesting TOPIC for the circumstances? (See RHH 3a, 3c-g.)	4.0 3.0 2.0 1.0 0.0 |-----|-----|-----|-----|-----|
2. Does this film have a clear, original, specific POINT (THESIS)? (See RHH 3a, 3h-l.)	4.0 3.0 2.0 1.0 0.0 |-----|-----|-----|-----|-----|
3. Does this film provide adequate SUPPORT (REASONING AND EVIDENCE) for its thesis? (See RHH 4.)	4.0 3.0 2.0 1.0 0.0 |-----|-----|-----|-----|-----|
4. Is this film well ORGANIZED so that the audience can follow its points and examples? (See RHH 3p-q)	4.0 3.0 2.0 1.0 0.0 |-----|-----|-----|-----|-----|
5. Does this film employ CLEAR, PRECISE LANGUAGE?	4.0 3.0 2.0 1.0 0.0 |-----|-----|-----|-----|-----|
6. Is this film factually ACCURATE and also FAIR, including recognizing reasonable objections? (See RHH 4f-h.)	4.0 3.0 2.0 1.0 0.0 |-----|-----|-----|-----|-----|
7. Is this film presented in a way that is ENGAGING to the audience?	4.0 3.0 2.0 1.0 0.0 |-----|-----|-----|-----|-----|
8. Is this film of a REASONABLE SIZE for the circumstances (10-15min)?	4.0 3.0 2.0 1.0 0.0 |-----|-----|-----|-----|-----|
9. Does this film clearly CREDIT OTHERS when their ideas and words are used?[2] (See RHH 7.)	4.0 3.0 2.0 1.0 0.0 |-----|-----|-----|-----|-----|
10. Does this film utilize editing software well and show significant EFFORT, CREATIVITY and TECHNICAL clarity when viewed?	4.0 3.0 2.0 1.0 0.0 |-----|-----|-----|-----|-----|

[1] This is adapted from a form put together by various LBC HPS faculty. Chapter and section references are to Frederick Crews, *The Random House Handbook*, sixth edition (New York: McGraw-Hill, 1992).

[2] Note that plagiarism is grounds for failure of the assignment and the course. If you're not sure what counts as plagiarism, ask early and often!

THE ELEMENTS OF A GOOD HYPOTHESIS

The American Heritage Dictionary defines hypothesis as "*a tentative explanation for an observation, phenomenon, or scientific problem that can be tested by further investigation*". A hypothesis is a possible answer to a question, from which predictions can be made and tested. There can be multiple hypotheses used to answer a single question and for each hypothesis, multiple predictions can usually be made.

The foundation for high quality, biological research is a good hypothesis. A good hypothesis is more than just an educated guess.

THE HYPOTHESIS SCORE CARD…

A good hypothesis must:

1.) explain how or why: provide a mechanism

2.) be compatible with and based upon the existing body of evidence.

3.) link an effect to a variable.

4.) state the expected effect.

5.) be testable.

6.) have at least two outcomes.

7.) have the potential to be refuted.

Hypotheses can be scored based on these elements. When considering a hypothesis, give one point for each of the elements. An *accomplished hypothesis* will have a score of 7. An *incomplete or developing hypothesis* will have a score of 5-6. A score below 5 is an *attempted hypothesis* or *not a hypothesis*. You should use this scoring procedure when developing your own hypotheses or when evaluating hypotheses of others.

Example Student Appeal

(student appealed the scoring of their answer to an exam question)

Name Removed

What is Mendel's Law of Segregation most closely associated with: Meiosis I or Anaphase I?

Claim:

- The law of segregation is most closely associated with Anaphase I.

Evidence:

- "Law of segregation states that paired chromosomes move to opposite nuclei." (Campbell Ch.3 pp36)

  Campbell, Heyer, Paradise. *Integrating Concepts in Biology*. 2014; ch.3 pp. 36

- "Homologous chromosomes separate in anaphase I…" (Campbell Ch.3 pp16)

  Campbell, Heyer, Paradise. *Integrating Concepts in Biology*. 2014; ch.3 pp. 16

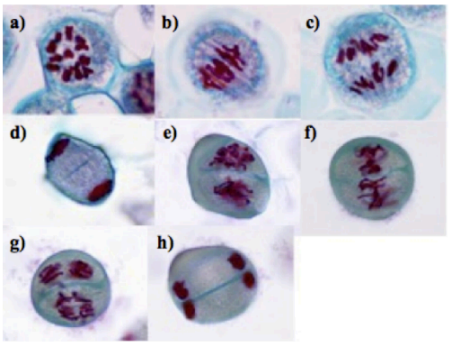
- 

  Figure 3.8 Egg formation in a lily plant with DNA stained red. (a) Parental diploid cell has undergone DNA replication. (b) Chromosomes condense and pair up with their matching chromosome. Each pair becomes closely intertwined, and then moves to the equator. (c) Paired chromosomes separate and migrate to opposite poles. (d) Cytokinesis separates the haploid nuclei. (e-f) In each cell, chromosomes are repositioned and then align on their respective equators. (g) Attached chromatids are separated and migrate to different poles. (h) Chromosomes coalesce inside nuclei and cytokinesis will produce four haploid cells.

  Figure 3.8:
  Campbell, Heyer, Paradise. *Integrating Concepts in Biology*. 2014; ch.3 pp. 15

- "The Law of Segregation states that each hereditary characteristic is controlled by two 'factors' (now called alleles), which segregate (separate) and pass into separate germ (reproductive) cells." (Hine & Martin)

  Hine, R. and E. Martin. A Dictionary of Biology. *Oxford University Press*. 6.

Reasoning:

- Meiosis I is the first half of meiosis that ends in two diploid cells, and is followed by meiosis II which ends in four haploid gametes. Gregor Mendel devised two laws to help understand this overall process of meiosis. These two laws are as follows: The Law of Segregation and The Law of Independent Assortment. In this case, we focused specifically on the law of segregation. This law is most specifically associated with anaphase I. To better illustrate this, one must understand what happens during anaphase I. In this stage, the homologous chromosomes separate and migrate to their opposite poles. As stated in our book, and in the Dictionary of Biology (published by Oxford University Press) it says that the law of segregation explains how the homologous chromosomes split and move to opposite nuclei, which is identical to the description of what happens in anaphase I. As shown by Figure 3.8 (found in Ch3 on page 15) the image labeled "C" illustrates anaphase I. In this image, the chromosomes are dyed red, which are shown splitting and moving to opposite sides of the cell. In conclusion, the Law of Segregation is specifically associated with anaphase I.

Draconian Contract

- **Purpose**

  To establish contractually binding ground rules for a team if the previously existing contract generated by the group is unclear and inadequately detailed.

- **Professional Agreement**

  As a member of this Research Team, I agree to do my part of the project—based on my designated Team Role (Pg.21). In addition to doing my part, I agree to putting in an equal work effort in order to pull my weight for the team. Also, I agree to bring an outstanding work ethic to the team—so that we may achieve a 4.0 level grade in the course. Therefore, I agree to take on all of the course work that a 4.0 level grade entails. Finally, I agree to treat my teammates with the upmost respect—by uphold all terms of this binding contract.

- **Clause I -Established Meeting and Communication Expectations:** Ground rules that will set up a known time for students to meet—thus allowing them to guarantee that additional out of lab hours will be put it for this course. In addition, to set up a known communication plan that includes the means of communication that will be utilized (with the preference included). Finally, establishing time and place to meet prior to the beginning of lab.

A. Team Meeting
   1. **As a team we will meet bi-weekly for laboratory.** The location of the meeting will be the East Lounge of East Holmes Hall.
      I. Sunday – 9am to 10am
         a. Prepare for the upcoming weeks lab.
         b. Discuss the assigned pre-readings.
         c. Formulate a plan for use of in lab hours.
            i. See Appendix for Plan Worksheet
         d. Observe Proposed Animal Communication Behavior
      II. Friday – 6pm to 7pm
         a. Discuss what was presented in lab that week.
            i. Lecture topic notes
            ii. Assignments
         b. Gather data from Observations
            i. Work on Manuscript and Documentary
               - Divide and Conquer using Team Roles (Pg.21)
         c. Formulate a plan for the next meeting
            i. See Appendix for Meeting Plan Worksheet
   2. **All members MUST attend the Team Meetings.**
      I. In the case where a member will be unable to attend, they must contact all members 24 hours in advance.
         a. Maximum Excused Absences = 2 meetings

II. If a member does not contact the team 24 hours in advance, or misses more than 2 meetings—**Go To Clause II.**

B. Team Communication
1. As a team we will communicate through the following means in order…
I. Cell Phone Group Message
a. ALL members will respond to a given message within an HOUR of message being delivered.
i. If a member does not follow the ground rules—**Go To Clause II.**
II. Team Email—using strictly @msu.edu
a. ALL members will respond to a given message within an HOUR of message being delivered.
i. If a member does not follow the ground rules—**Go To Clause II.**

C. Pre Lab Meetings
1. ALL members will show up to each scheduled lab class meeting
I. Prior to lab beginning, ALL members will meet 10 minutes before regular class, and 15 minutes before class when important assignments are due (i.e. Manuscripts)
a. Location = Directly Outside of C4 Holmes Hall

❖ **Clause II -Meeting and Communication Breach of Contract Incidents:** Ground rules to establish a known protocol concerning how the team will deal with a breach of contract. A breach of contract, by definition, is time when a binding agreement has not been honored by one or more parties involved in the agreement. When breach of contract has occurred, a statement must be filed with the LB144 teaching assistant (TA) that states how the contract was breached and who has not honored the agreement.

A. Not Attending Meetings
1. As stated in Clause IA Paragraph 2 Subset I—Any member who is not able to attend a scheduled mandatory meeting must contact his/her team 24 hours in advance. Also, any member is allowed 2, and ONLY 2, excused absences from the mandatory meetings. While Clause IC Paragraph 1 states the team will meet prior to lab.
I. If any member fails to uphold this portion of the agreement a breach of contract has occurred.

a. First Incident Claim
i. If any member fails to contact the team 24 hours in advance of missing a mandatory team meeting or has missed a total of 3 team meetings or has missed a pre lab meeting, the team must declare this breach of contract BOTH in the CATME feedback surveys as well as in writing. The CATME feedback surveys will occur regularly throughout the

semester. A hard copy of written notice must be given directly to the offender(s) and a hard copy also provided to the TA in lab section. The hard copy allows the LB144 Teaching Team to date and file the initial claim for record.

- The form for breach of contract written claims can be found in the Appendix.
  - A new clam is not permitted for 2 weeks.

b. Second Incident Claim

i. If any member fails to contact the team 24 hours in advance of missing a mandatory team meeting (for the Second Time) or has missed a total of 4 team meetings or has missed a pre lab meeting, the team must declare this breach of contract BOTH in the CATME feedback surveys as well as in writing. The CATME feedback surveys will occur regularly throughout the semester. While the writing must be given directly to the offender(s) and a hard copy provided to the TA in class. The hard copy allows the LB144 Teaching Team to date and file the claim for record.

- The form for breach of contract written claims can be found in the Appendix.

ii. In addition, the team and TA must arrange a date for a Face-to-Face Encounter—in which ALL team members MUST attend.

- Failure to attend the Face-to-Face encounter will result in immediate dismissal of the member(s) from the team—**Go To Clause IV.**
  - A new claim is not permitted for 2 weeks.

c. Third (and Final) Incident Claim

i. If any member fails to contact the team 24 hours in advance of missing a mandatory team meeting (for the Third Time) or has missed a total of 5 team meetings or has missed a pre lab, the team must declare this breach of contract BOTH in the CATME feedback surveys as well as in writing. The CATME feedback surveys will occur regularly throughout the semester. While the writing must be given directly to the offender(s) and a hard copy provided to the TA in class. The hard copy allows the LB144 Teaching Team to date and file the claim for record.

- The form for breach of contract written claims can be found in the Appendix.

ii. In addition, a hard copy of the claim must be provided to the professor (Dr. Luckie). The professor hard copy must comprehensively detail all past events, which led to all prior claims, and the current event.
- The *professor form* for breach of contract written claim can be found in the Appendix.

iii. In addition, the team must contact the professor to setup a mandatory Face-to-Face encounter—in which ALL members must attend.
- Failure to attend the Face-to-Face encounter will result in immediate dismissal of the member(s) from the team—**Go To Clause IV.**
- If the professor agrees with the claims…
  - Divorce Clause proceedings begin immediately and can only be completed 2 weeks after the professor meeting.
- If the professor does not agree with the claims…
  - The team will remain with all members in good standing. Unless the team decides to initiate the Divorce clause themselves—which at that point can only be completed 4 weeks after the professor meeting.

B. Not Following Communication Norms

1. As stated in Clause IB—any member of the must communicate in a timely and respectable fashion. The time period for all response must be within an hour of the original message.
   I. If any member fails to uphold this portion of the agreement a breach of contract has occurred.

a. First Incident Claim

i. If any member fails to respond to a group message within an hour, the team must declare the breach of contract to the LB144 TA. In order for the team to successfully submit a claim they must provide a hard copy to the offender(s) and provide a hard copy to the TA, along with a screenshot proving a lack of communication, during class. The hard copy allows the LB144 Teaching Team to date and file the initial claim for record.
- The form for breach of contract can be found in the Appendix.
  - A new claim is not permitted for 2 weeks.

b. Second Incident Claim

i. If any member fails to respond to a group message within an hour, the team must declare the breach of contract to the LB144 TA. In order for the team to successfully submit a claim they must provide a hard copy to the offender(s) and provide a hard copy to the TA, along with a screenshot proving a lack of communication, during class. The hard copy allows the LB144 Teaching Team to date and file the claim for record.
   - The form for breach of contract can be found in the Appendix.

ii. In addition, the team and TA must arrange a date for a Face-to-Face encounter—in which ALL members must attend.
   - Failure to attend the Face-to-Face Encounter will result in immediate dismissal of the member(s) from the team—**Go To Clause IV.**
     - A new clam is not permitted for 2 weeks

c. Third (and Final) Incident Claim

i. If any member fails to respond to a group message within an hour, the team must declare the breach of contract to the LB144 TA. In order for the team to successfully submit a claim they must provide a hard copy to the offenders(s) and provide a hard copy to the TA, along with a screenshot proving a lack of communication, during class. The hard copy allows the Lb144 Teaching Team to date and file the claim for record.
   - The form for breach of contract can be found in the Appendix.

ii. In addition, a hard copy of the claim must be provided to the professor (Dr. Luckie). The professor hard copy must comprehensively detail all past events, which led to all prior claims, and include all screenshots provided to the TA.
   - The *professor form* for breach of contract can be found in the Appendix.

iii. In addition, the team must contact the professor to setup a mandatory Face-to-Face encounter—in which ALL members must attend.
   - Failure to attend the Face-to-Face encounter will result in immediate dismissal of the

member(s) from the team—**Go To Clause IV.**

- If the professor agrees with the claims…
  - Divorce Clause proceedings begin immediately and can only be completed 2 weeks after the professor meeting.
- If the professor does not agree with the claims…
  - The team will remain with ALL members in good standing—in which the professor drafts a new communication clause. Unless the team decides to initiate the Divorce clause themselves—which can only be completed 4 weeks after the professor meeting.

❖ **Clause III- Break of Contract Grievances related to *Performance Expectations*:** Ground rules to establish a known protocol concerning how the team will deal with grievances. A grievance, by definition, is a feeling of being wronged (or treated unfairly)—in which a statement is made stating HOW one was wronged and WHY one is not satisfied with an occurrence. Thus, in LB144 a grievance can occur from the following situations listed in this clause.

A. Performance Expectations

1. **As stated by the Professional Agreement section of this contract—ALL members who sign this document agreed to** …
   I. Do their part based on Team Roles (Pgs. CoursePack)
   II. Put forth Equal Work Effort.
   III. Work Ethic equivalent to achieve a 4.0-level grade.
      a. Completing all work needed for a 4.0-level grade.
   IV. Respect all terms of the contract.

2. **Failure to uphold any of the four previously stated Professional Agreements may lead to grievances within the team.**

I. First Incident Claim

a. If an individual feels any of the Professional Agreements are not being fulfilled, they must declare it in the CATME feedback survey, provide a written hard copy for the TA, and have an interventional meeting (5 minutes maximum) in lab. The hard copy allows the LB144 Teaching Team to date and file the initial grievance for record.
   i. A new grievance is not permitted for 2 weeks.
      - The grievance claim form can be found in the Appendix.

II. Second Incident Claim

a. If an individual feels any of the Professional Agreements are not being fulfilled, they must declare it in the CATME feedback survey, provide a written hard copy for the TA, and have an interventional meeting (5 minutes maximum) in lab. The hard copy allows the LB144 Teaching Team to date and file the initial grievance for record.
   i. A new grievance is not permitted for 2 weeks.
      - The grievance claim form can be found in the Appendix.

b. In addition, the team and TA must arrange a date for a Face-to-Face Encounter—in which ALL members must attend.
   i. Failure to attend the Face-to-Face Encounter will result in immediate dismal of the member(s) from the team—**Go To Clause IV.**
      - A new grievance is not permitted for 2 weeks.

III. Third (and Final) Incident Claim

a. If an individual feels any of the Professional Agreements are not being fulfilled, they must declare it in the CATME feedback survey, provide a written hard copy for the TA, and have an interventional meeting (5 minutes maximum) in lab. The hard copy allows the LB144 Teaching Team to date and file the initial grievance for record.
   i. The grievance claim form can be found in the Appendix

b. In addition, a hard copy of the claim must be provided to the professor (Dr. Luckie). The professor hard copy must comprehensively detail all past events, which lead to prior grievances, and the current event.
   i. The professor grievance claim form can be found in the Appendix.

c. In addition, the team and professor must arrange a date for a Face-to-Face Encounter—in which ALL members must attend.
   i. Failure to attend the Face-to-Face Encounter will result in immediate dismal of the member(s) from the team—**Go To Clause IV.**
   ii. If the Professor agrees with the claim…

- Divorce Clause begins immediately and can only be completed 2 weeks after the professor meeting.

iii. If the Professor does not agree with the claim…
- The team will remain with ALL members in good standings. Unless the team decides to initiate the Divorce Clause themselves—which can only be completed 4 weeks after the professor meeting.

❖ **Clause IV: *Prenuptial* agreement basis for "Divorce" proceedings.** In any case where a team feels that the environment is no longer suitable—and a member is "fired" or decides to leave. The intellectual property of the group must be distributed, which is outlined in this clause.

A. **QUITTING** In the case where a Team Member decides to willingly separate from their group…
   1. ALL Intellectual Property is maintained within the Team.
      I. Deciding to separate from your team is equivalent to deciding to separate from a company. As is the case with a corporation, all products you created are retained by the company you departed. You have no rights to those products. All you the intellectual property—including your own work is retained by the remained group members. You therefore must start the lab over from zero as a group of 1 person. You cannot plagiarize your own prior writing, use your prior citations, methods, experimental design etc.
         a. In short, you must start an entirely NEW and DIFFERENT research project.

B. **FIRING** In the case where a Team Member is fired from the Team…
   1. ALL Intellectual Property is maintained with the Member of the Team who was fired.
      I. Deciding to fire a member is equivalent to the team as a whole deciding to separate from the individual and all of their intellectual property. The party who was fired in this case gains the rights of the corporation when all the other group members quit. The remaining group members must, therefore, start from the lab over from Square 1 and cannot use/plagiarize their own prior writing, prior citations, methods, experimental design etc

C. **MISC/OTHER** In any case of Divorce, one party maintains all intellectual property and the other party loses all intellectual property—having to start over. Depending on the week in which the divorce is finalized, all graded assignments prior to the divorce will remain. However, the party that is required to start over will have lost the opportunity to have the feedback from these assignments for their NEW research project.

1. Example
    I. A team member decides to separate from the team after the Proposal and Half-Draft of the Research manuscript. As stated previously, this team member will be giving up all rights to all intellectual property of the team. In addition, all grades for these assignments will be maintained after the divorce. However, since the individual member must start a new and entirely different research project they have lost the opportunity to receive feedback at either the Proposal and Half-Draft stage of authorship of manuscript. Therefore, the first official graded assignment under this new research project will be the Draft1 of the manuscript.

D. **ONLY SOLO** In the case of any form of divorce—any party who either separates from the group, or is fired, MUST finish the semester as an INDIVIDUAL. Forming a new group or joining a pre-existing group is NOT permitted.

Sunday Team Meeting Plan Worksheet

Name(s): _____

Section: _____ Date: _____

Course-Pack Readings (Pages): _____

Reading Summary:

_____

_____

_____

_____

Observations:

_____

_____

_____

_____

Detailed Plan for Use of In-Lab Hours:

_____

_____

_____

_____

_____

_____

TA Signature: X_____

Friday Team Meeting Plan Worksheet

Name(s): _____

Section: _____ Date: _____

Homework:

_____

_____

Describe Lecture Topic:

_____

_____

_____

_____

Manuscript & Documentary—What was Worked On and By Who?

_____

_____

_____

_____

Next Meeting Agenda:

_____

_____

_____

_____

TA Signature: X_____

TA Breach of Contract Form

Names: _____

Offender(s): _____

Claim: _____ First _____ Second _____ Third

Reason: _____Team Meeting _____ Communication _____ Pre-Lab

Date Occurred: _____

Description:

_____

_____

_____

_____

_____

_____

Section: _____ TA: _____

Student Signature: X _____

TA Signature: X _____

Date Issued: _____

Next Claim Issue Date (2 weeks): _____

Professor Breach of Contract Form

Names: _____

Offender(s): _____

Date Occurred: _____ Section: _____

Description:

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Student Signature: X _____

Date Issued: _____

Next Claim Issue Date (2 weeks): _____

TA Grievance Form

Names: _____

Offender(s): _____

Claim: _____ First _____ Second _____ Third

Date Occurred: _____

Description:

_____

_____

_____

_____

_____

_____

Section: _____ TA: _____

Student Signature: X _____

TA Signature: X _____

Date Issued: _____

Next Claim Issue Date (2 weeks): _____

Professor Grievance Form

Names: _____

Offender(s): _____

Date Occurred: _____ Section: _____

Description:

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Student Signature: X _____

Date Issued: _____

Next Claim Issue Date (2 weeks): _____

Divorce Form

Name: _____

Divorce Type: (Please Check One)

_____ I, _____, willingly have decided to separate from my group.

_____ As a team, we have decided to fire _____ .

Reason:

_____

_____

_____

_____

_____

_____

Date Issued: _____ Effective Date: _____

Signature of ALL Team Members:

X _____

X _____

X _____

X _____

Signature of TA:

X _____

Signature of Professor:

X _____

Student Laboratory Notebook