MACUB

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Saturday, October 29, 2016

SUNY at Old Westbury

Old Westbury, New York

Conference Theme

The Dance of the Genes: From Cancer to Conservation

Keynote Speakers

Jill Bargonetti

Unraveling the Creativity of Choreographing Cancer Genomics

will be presented by

And

Mary E. Blair

How Can Genetics Inform Biodiversity Conservation? Opportunities and Pitfalls

will be presented by

Instructions for Authors

IN VIVO is published three times yearly during the Fall, Winter, and Spring. Original research articles in the field of biology in addition to original articles of general interest to faculty and students may be submitted to the editor to be considered for publication. Manuscripts can be in the form of a) full length manuscripts, b) mini-reviews or c) short communications of particularly significant and timely information. Manuscripts will be evaluated by two reviewers.

Articles can be submitted electronically to *invivo@mec.cuny.edu* or mailed as a printed copy (preferably with a diskette that contains the file) to the Editorial Board at Medgar Evers College. All submissions should be formatted double spaced with 1 inch margins. The title of the article, the full names of each author, their academic affiliations and addresses, and the name of the person to whom correspondence should be sent must be given. As a rule, full length articles should include a brief abstract and be divided into the following sections: introduction, materials and methods, results, discussion, acknowledgments and references. Reviews and short communications can be arranged differently. References should be identified in the text by using numerical superscripts in consecutive order. In the reference section, references should be arranged in the order that they appeared in the text using the following format: last name, initials., year of publication. title of article, journal volume number: page numbers. (eg. - [1]Hassan, M. and V. Herbert, 2000. Colon Cancer. *In Vivo* **32:** 3 - 8). For books the order should be last name, initial, year of publication, title of book in italics, publisher and city, and page number referred to. (eg. - Prosser, C.L., 1973. *Comparative Animal Physiology*, Saunders Co., Philadelphia, p 59.). Abbreviations and technical jargon should be avoided. Tables and figures should be submitted on separate pages with the desired locations in the text indicated in the margins.

In This Issue:

Save the Date
The 2016 MACUB Conference will be at SUNY at Old Westbury
October 29, 2016

All in our campus community are excited and honored to host the 49th annual MACUB Conference and we look forward to welcoming you to our Long Island campus this fall! Stimulating dialogue among those with a passion for biology is a key foundation of MACUB and we at SUNY Old Westbury look forward to sharing our passion with you as we seek to provide all in attendance, faculty and student alike, with a greater appreciation for all that surrounds us.

Located in the nation's first suburbs, just 20 or so miles from the center of Manhattan, ours is a 604-acre campus that offers a serene, sequestered setting perfect for collaboration and the pursuit of knowledge. We believe it to be the perfect location for this conference's topic: "The Dance of the Genes: From Cancer to Conservation."

Growth in the Natural Sciences Departments at SUNY Old Westbury remains at a fast pace, with the number of majors in Biological Sciences, Biochemistry, Chemistry and Health and Society outpacing all other programs within our campus community. In addition, the science faculty, with undergraduate student-researchers by their sides, is at work investigating various aspects of developmental biology, microbial ecology, genomics, neurobiology, cancer biology, and antibiotic resistance. College-wide, our students learn from a faculty of intellectuals, 83% of whom hold terminal degrees in their fields and who, through their research and scholarly pursuits, are credited with cutting edge discoveries and findings regularly.

As an academic institution, SUNY Old Westbury remains committed to ensuring that students from diverse backgrounds and socioeconomic strata have the opportunity to pursue their collegiate dreams. The College has over the past several years welcomed its largest entering classes on record and seen more than 1,000 students annually graduate with their degrees in hand. These students study in our more than 40 undergraduate degree programs and 16 graduate degree programs.

The success of SUNY Old Westbury has been recognized widely, with the college being named by U.S. News and World Report among the most diverse liberal arts colleges in America, a "Military Friendly School" for its programs to support those in the armed services, and most proudly, by the U.S. President's Higher Education Community Service Honor Roll for seven consecutive years.

We look forward to serving as host for the 2016 Fall MACUB Conference and to welcoming all of you on October 29.

Fernando Nieto, Ph.D.
Chairman, Biological Sciences Department
SUNY Old Westbury

Keynote Speakers

Jill Bargonetti, Ph.D, a renowned cancer researcher, earned her B.A. at SUNY College at Purchase, her Ph.D. at New York University and did postdoctorate work at Columbia University. She serves as chair of the molecular, cellular, and development subprogram in the Ph.D. Program in Biology at the Graduate Center and as professor of biological sciences at Hunter College. Since 1994, she has been running the Bargonetti Lab at Hunter College, where her team is using genetically engineered tools to research breast cancer. A current standing member of the Tumor Cell Biology study section grant review panel for the National Institutes of Health (NIH), Bargonetti has published her research in prestigious scientific journals, has received research grants from the National Science Foundation and the NIH, and is an innovator in the education of minorities in science. Her awards include a Young Investigator Award (given by the mayor of New York City); the 1998 New York Voice Award (given to those who have made a significant improvement to the quality of life in New York City); the 1997 Kathy Keeton Mountain Top Award from the New York branch of the NAACP; the Presidential Early Career Award for Scientists and Engineers, from President Bill Clinton in 1997; the New York City Mayor's Award for Excellence in Science and Technology; the Outstanding Woman Scientist Award from the Association for Women in Science; the Breast Cancer Research Foundation Award; and the 2005 NYU Graduate School of Arts and Science Alumnae Achievement Award. Bargonetti was profiled by *Working Mother* magazine in December 2004 as one of the nation's "Stellar Moms."

Mary E. Blair, is the Assistant Director for Research and Strategic Planning at the Center for Biodiversity and Conservation at the American Museum of Natural History (AMNH). She is also Affiliated Faculty at Columbia University, the New York Consortium in Evolutionary Primatology, and the Richard Gilder Graduate School at the AMNH. As a conservation biologist focusing on primates, her research integrates spatial modeling and molecular genetics to understand the evolutionary processes that generate biodiversity and the influence of environmental variability on evolutionary divergence. She also explores how knowledge of evolutionary processes can inform conservation planning and the spatial prioritization of conservation actions.Most recently, she is investigating the diversity of slow lorises in Vietnam and the patterns, scales, and drivers of illicit trade in these and other animals through an NSF Science, Engineering, and Education for Sustainability (SEES) Fellowship. In 2013, she co-edited *Primate Ecology and Conservation: A Handbook of Techniques,* published by Oxford University Press. Her blogs for the *New York Times'* Scientist at Work and AMNH's From the Field have reached a global audience. Dr. Blair is Affiliated Faculty at Columbia University and at the Richard Gilder Graduate School at the AMNH, and is President of the AMNH Chapter of the Association for Women in Science. She received her B.A. from Swarthmore College in 2005 and her M. Phil and Ph.D. in Evolutionary Primatology from Columbia University in 2011.

Effectiveness of Problem Based-Learning on Student Performance in Mastering Difficult Concepts in an Introductory Anatomy and Physiology Course

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Abstract

The aim of this study was to assess the impact of the problem-based learning method (PBL) on student performance in an introductory Anatomy and Physiology course (A&P). Students who lack critical problem solving skills face significant challenges in A&P courses. Their ability to retain and apply the concepts that they have learned in A&P to real life and clinical trial is tremendously important. This holds especially true for students in pursuit of the allied health fields. This study focused on the impact of the problem-based learning method on: improving the critical and analytical thinking skills of students; increasing the students' ability to make connections between various topics and disciplines; enhancing the students' level of engagement; and supporting the relevance of content taught in the classroom to real life. The PBL activities that were presented to the students acted as a stimulus for the development of their critical thinking skills and the problem-solving abilities necessary for application in the real world. Overall, this study found that the use of the PBL method in introductory A&P classrooms had a positive impact on student performance, increasing the course's pass rate, improved classroom engagement and academic performance among the students.

Introduction

Identifying the Problem

Critical and creative thinking, inquiry and analysis, teamwork and problem solving "practiced extensively, across the curriculum, in the context of progressively more challenging problems, projects, and standards for performance to engage complex problems and questions and to ensure that students develop facility in evidence-based inquiry, analysis, and decision making" have been identified by The Association for American Colleges and Universities[1,2] as some of the most integral college education skills of the twenty-first century[3]. A common problem experienced on campuses across America is that most of the students enrolled in the gateway science courses, particularly introductory Anatomy and Physiology (A&P), which is a prerequisite for many allied health science programs such as nursing, radiation technology, and nuclear medicine, lack these skills.

Students often indicate that in addition to their lack of critical and analytical thinking skills, they lack basic scientific knowledge, and often experience difficulty in establishing connections across various topics in multiple disciplines, which only increases the difficulty of the course. Students taking A&P have consistently demonstrated having a tough time understanding and retaining the information presented, and they struggle with the application of this material in

clinical practice[4-6]. A majority of our students struggle daily to manage their time and availability due to their busy working schedules and familial obligations; juggling between reading a solid portion of the textbook and understanding the concepts presented in lectures.

Addressing the Issues

In an effort to resolve some of the outlined issues that have impacted our student body, the following steps were taken: First, I identified the main learning objectives of the introductory A&P course. Second, the concepts which students struggled the most with, as identified through the compilation of correlated data from previous semesters, were singled-out. Last, a number of different teaching methods were researched in an effort to best address the difficulties students were having with the previously identified learning objectives.

It was through the use of this process that I was able to identify problem-based learning (PBL) as a method with which the difficulties facing A&P students could be addressed and the academic performance of those who had been struggling with introductory Anatomy & Physiology could be improved. Therefore, the purpose of this study was to investigate "Does the use of PBL learning method increase students' overall success in my introductory A&P courses"

The teaching of A&P has traditionally been conducted through lectures and practicals[6]. The PBL method places greater emphasis upon student engagement. PBL activities combine the fundamental application of real-world experience with the theory of academic classrooms and promote an understanding of the area of study[7]. The educational method in PBL has been described as student-centered where the educator's role is to facilitate, guide and monitor the learning process. In the PBL process, students become self-regulated learners, relying upon previous knowledge to guide their thought process and foster their educational growth. This engages the students in a manner of learning that they will continue to utilize throughout their careers[8].

PBL provides educators with an opportunity to expose students to real-world problems which are meant to act as a stimulus for developing the critical thinking skills and problem-solving abilities that are needed for the conduct of, and application in, their careers. The use of PBL would also improve the writing skills of struggling students by challenging them to effectively compile their critical thinking skills and problem-solving abilities into formulated responses. PBL provides the students with an opportunity to master the difficult scientific concepts posed in this course, or in any field, by allowing them to exercise their critical thinking skills via solving problems[9]. This method would be an instrumental tool in aligning the PBL-enhanced A&P course with the professional work students are expected to engage in upon graduation.

Implementing the Solution

This report summarizes my findings from the use of the PBL method to address the difficulties faced by students in introductory A&P for the purpose of increasing their academic performance. The course material and the previously identified topics that the students struggled most with were addressed through the use of real life scenarios that specifically emphasized the relevance of the material and content to real life situations and those in clinical practice.

I created a number of problem-based case studies that were purposefully designed to resemble real life scenarios and encourage the students' critical thinking skills and problem solving abilities. These case studies also tested the students' retention of qualitative and quantitative knowledge on the topics that they struggled most with. The areas of study that were incorporated into these case studies included, but were not limited to, the chemistry of life, homeostasis, cell/ membrane transport/solutions, the integumentary system/tissues, and the nervous system/action potential. These problem areas were identified through a formal analysis of correlated data gathered from the departmental common final exams from previous semesters of introductory A&P courses that I have taught.

Two examples of these problem-based learning activities (i.e. case studies) are provided below to demonstrate the increase that the use of PBL case studies in an introductory A&P course had on student performance.

Methods

This study was conducted at Bronx Community College of the City University of New York over the course of six different semesters between 2007 and 2012. In total, twelve sections of first-semester introductory Anatomy & Physiology courses were studied. The students participating in this study were not provided with any advance notice of the two separate sections for this course; PBL-enhanced versus non-PBL. The students' selection of their course was not influenced by the existence of this study to the extent that this was possible. Over the period of the referenced six semesters, the academic performance of 152 students who completed the course were examined. Students who received non-academic grades ("Incompletes" and all variation of "Withdrawals") were not taken into consideration of the outcome. This was done to avoid the consideration of outside factors, such as work schedules and familial obligations that may have negatively impacted a students' ability to participate in the course through completion, that would have affected the research question underscoring the degree of change that adding PBL to the teaching of introductory A&P would have upon student performance. The selection of which course would be taught through the use of the PBL method and which course would be taught through traditional lectures was conducted at random. All sections of the course considered in this study were taught by myself. Of the 152 students, 75 students have attended the PBL-enhanced sections, accounting for approximately 50% of the overall student performance studied.

The course is a 4-credit course, comprising of three laboratory hours and three lecture hours per week. The prerequisites for this course are Elementary Algebra (MTH 05), and Reading and Study Skills (RDL 02) and Developmental Writing II (ENG 02) if required. The course primarily serves students preparing to enter Allied Health fields, including Nursing, Radiation Technology, Nuclear Medicine, and Nutrition.

Identifying the Learning Objectives of the Course

In order to encourage the integration of knowledge, basic science should be presented in the context of a clinical scenario[10]. This is exactly where PBL excels. The clinical scenarios that are

presented to the students lead them to a particular area of study for the purpose of encouraging them to gain a better understanding of the learning objectives that had previously been identified. To achieve the best results, first, the learning objectives of the course were identified (Table 1).

Table 1. Learning Objectives of the Introductory A&P Course

1	To learn the fundamental concepts and principles of anatomy and physiology and its clinical applications.
2	To learn anatomical terms, chemistry, the basic structure of the cells and cell specialization, tissue histology, the location, structure and functions of the systems.
3	To learn how to explain complex physiological mechanisms in writing and verbally.
4	To learn how to properly label major anatomical structures.
5	To develop scientific problem solving skills, critical thinking skills, and writing skills.
6	To learn how to read primary research articles and verbally explain/present the methods and major findings and relate the information to the class content.
7	To understand basic scientific principles, the role of biology in modern society, clinical applications, malpractice, current issues and developments in biology.

Design of the PBL Activities

The problem-based case studies had purposefully been designed to add real-world relevancy to the course in the areas which students struggled the most. Students had a tendency to exhibit weak quantitative literacy skills in addition to the difficulty they experienced in the comprehension of complex concepts. The case studies were developed with the intention that the content would be relevant to their learning objectives, would incorporate real world and clinical applications, and improve their general scientific knowledge. Each PBL case study also identified the correlative Gen-Ed proficiencies and the learning outcomes of that specific topic. Two of the case studies particularly focused on using a combination of these skills, including a requisite calculation for the interpretation of data in understanding topics such as macromolecules and membrane transport -solutions.

Implementation of the PBL Activities into the Classroom

Topics relevant to the case studies were emphasized during lectures and students were asked to complete a pre-test of the topics discussed. This pre-test was only offered to the students enrolled in the PBL-enhanced sections of A&P as the purpose of this was to directly measure the impact that the use of the PBL method had upon the students' performance. The students were then asked to work on the case studies independently and, subsequently, in small discussion groups within the classroom. The students were provided with a list of the previously identified Gen-Ed learning objectives along with the outcomes for each case study (Tables 2 and 3).

A majority of the PBL case studies comprised of two separate stages. The questions in the first stages focused on understanding the key points relevant to the topic, and the questions in the second stages focused on the application of the topic to real life scenarios. The students were given 20-25 minutes in each stage for conducting independent, self-directed study. They subsequently returned to their groups to discuss and sharpen their acquired knowledge for another 15-minute period. Then, all of the assignments were collected from the students and graded with instructive comments. Interactive discussions were also held about the concepts that were

Table 2. PBL case study based on liquid chemical solutions and membrane transport

CASE STUDY 1: DANIELLE'S CELLS

Gen-Ed objectives:

Scientific Method: Students are expected use the scientific method to understand membrane transport and its relevance in physiology.

Reasoning and Analysis: Students are expected to use reasoning and analysis to evaluate, interpret and analyze the problem. The questions in Stage 1 require students to have knowledge of membrane transport, different types of liquid chemical solutions, and the effects of these solutions on cell physiology.

Learning Outcomes:

At the completion of this case study, students are expected to:

Describe the intravenous saline fluid and its composition;
Explain the effects of different fluids (i.e. liquid chemical solutions) on cell physiology;
Explain the relationship between membrane transport and tonicity;
Describe possible treatment strategies; and,
Explain the importance of maintaining ethics within the workplace.

ACTIVITY: STAGE 1

Melissa, a nursing student, lives at home with her parents and two younger siblings. Over the past couple of days, she had noticed that her mother, Danielle, who had been spending an abundant amount of time in the garden, appeared withdrawn, unsteady and complained of feeling weak and tired. Melissa, who then took her mother's blood pressure, contacted their family physician after she noticed that the blood pressure reading was markedly low, 75/52. Dr. Singh suggested that Melissa take her mother to the local hospital and receive treatment. He stated that her symptoms may be a sign of dehydration.

At the hospital, after being checked in and attended to by a physician, Danielle was administered an intravenous saline by the nurse. Approximately fifteen minutes later, Danielle complained of having difficulty breathing and Melissa noticed that her mother's skin has started to turn blue. Melissa immediately paged the nurse, who took another blood pressure reading, and found that Danielle's blood pressure was 180/95.

Discussion Questions

What is intravenous saline? What kind of solution is it?
Why was Danielle treated with intravenous saline?
Given what we know of the case study's history, what might be the possible cause of these symptoms?
What process is taking place in Danielle's cells?
How would you explain the physiological change in the shape of her cells?
What treatment would you immediately administer to reverse her condition?

STAGE 2

The nurse called the doctor immediately. The doctor realized that the nurse made a terrible mistake by using the wrong intravenous solution. After several hours of treatment and recovery Danielle finally felt better. Danielle and her family are upset and consider filing a lawsuit.

Discussion Questions

Whose fault is the error in treatment Danielle received? (the nurse, or the hospital)
How did this case study impact your thoughts on work ethics?

covered in those case studies during the lectures. Finally, students were given a post-test based on that activity. Again, the post-test was only offered to the students attending the PBL-enhanced section of the introductory A&P course.

Two Examples of the PBL Activities

The use of PBL case studies played three critical roles in increasing student performance: 1) The in-class discussions of scenarios with real-world applications served to encourage students to access

Table 3. PBL case study based on macromolecules

CASE STUDY 2: THE NUTRITION CLASS

Gen-Ed objectives:

Scientific Method: Students are expected to use the scientific method to identify the organic molecules and the energy contained within their daily diet. Students are also expected to calculate the energy they get out of their daily diet.

Reasoning and Analysis: Students are expected to use reasoning and analysis to evaluate, interpret and analyze the problem. The questions in Stage 1 and 2 require that students have knowledge of organic molecules.

Quantitative Literacy: Students are expected to use mathematical methods to calculate the percentages of total steroids, fatty acids, simple sugars and the calories they contain.

Learning Outcomes:

At the completion of this case study, students are expected to:

Identify the different types of carbohydrates, proteins, and lipids.
Identify the different types of carbohydrates, proteins, and lipids in different food groups.
Calculate the percentage of the total fatty acid and steroids of total fat, and the simple sugars in the breakfast cereal bar.
Explain the differences between simple sugars, starches, and fiber.
Calculate the portion the calories from fat, protein and carbohydrate in the bar.
Develop an understanding of healthy eating habits and portion size control.

ACTIVITY: STAGE 1

Todd, a high school junior and aspiring dietician, was very excited about his new science class on nutrition. Over the course of his first week in class, Todd read about as much as he could from the new textbook. At home, he wanted to put his new found knowledge to good use and decided to analyze the organic and molecular structure of the foods that his family had been eating.

For breakfast, his father had a cup of black coffee, a plain bagel with cream cheese, a glass of orange juice and grabbed a breakfast cereal bar.

For lunch, his younger brother ate a turkey breast sandwich with tomatoes, lettuce and mayonnaise on whole wheat bread. He washed it down with a can of orange soda.

At *dinner time*, Todd's mother enjoyed a bowl of pasta with a creamy alfredo sauce and drank one glass of red wine.

Discussion Questions

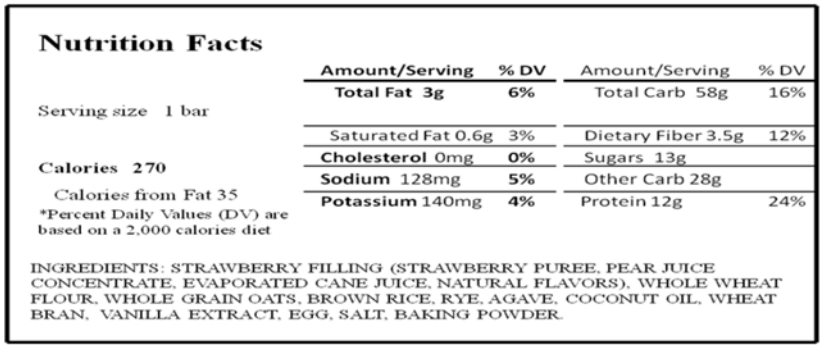
List all of the foods which contain carbohydrates.
Develop a rule to help you identify foods that contain carbohydrates.
How are carbohydrates normally created (i.e., what organism makes them)?
List all of the foods that contain lipids.
List all of the foods that contain proteins.

STAGE 2

A couple of weeks into the school year, the school's track & field coach asked the school's nutritionist to discuss the importance of healthy eating habits with the entire team. The nutritionist asked the team about their eating habits. As the team consisted of high school students, not many of them had given much thought about this prior to that day.

The team confessed they had a habit of stopping by the local fast food restaurant after practice. The nutritionist advised the athletes to minimize their fast food intake and pay extra attention to the portions of their meals. The nutritionist warned the students about the dangers of fast food and instructed them to read the nutritional fact content from the labels of the food they purchased so as to improve their performance. The nutritionist also showed them a couple of examples from various products. Todd, a member of the track & field team, suggested they review the nutritional fact content of the breakfast cereal bar he kept in his gym bag as a snack.

Analyze the Breakfast Cereal Bar food label below and then answer the questions that follow.



Nutrition Facts

Serving size 1 bar

Calories 270

Calories from Fat 35

*Percent Daily Values (DV) are based on a 2,000 calories diet

Amount/Serving	% DV	Amount/Serving	% DV
Total Fat 3g	**6%**	Total Carb 58g	16%
Saturated Fat 0.6g	3%	Dietary Fiber 3.5g	12%
Cholesterol 0mg	**0%**	Sugars 13g	
Sodium 128mg	**5%**	Other Carb 28g	
Potassium 140mg	**4%**	Protein 12g	24%

INGREDIENTS: STRAWBERRY FILLING (STRAWBERRY PUREE, PEAR JUICE CONCENTRATE, EVAPORATED CANE JUICE, NATURAL FLAVORS), WHOLE WHEAT FLOUR, WHOLE GRAIN OATS, BROWN RICE, RYE, AGAVE, COCONUT OIL, WHEAT BRAN, VANILLA EXTRACT, EGG, SALT, BAKING POWDER.

Discussion Questions

What percentage of the total fat in the bar comes from fatty acid?
What percentage of the total fat in the bar is a steroid?
What percentage of the carbohydrates in the bar is from simple sugars?
What are the differences between simple sugars, starches, and fiber?
Use this Breakfast-Cereal Bar label to find all the ingredients that are carbohydrates of the following classes:
- Simple sugars
- Complex carbohydrates (i.e. similar to starches)
- Complex carbohydrates that contain fiber

What portion of the total calories is derived from fat, protein, and carbohydrate?

and apply relevant prior knowledge. 2) The case studies engaged students, stimulating their interests and motivating them to learn. And, 3) the problem-based learning method established a context for learning similar to that in which the knowledge would be required of the student for use in the future[11].

As for my case studies, consider a topic from Introductory Chemistry (For example, the importance of using liquid chemical solutions in the medical field). During my initial years of teaching this topic and its applications, I realized that students experienced difficulty in comprehending the importance of liquid chemical solutions (i.e. intra-venous solution, etc.) and their applications in the medical field(s). After a number of attempts, I began to use the problem based learning method as a vehicle for the promotion of critical thinking and designed a case study which assisted the students in gaining a better understanding of liquid chemical solutions, their applications in the real-world, and their importance in the practice, or malpractice, of medicine.The two stages of this case study brought together multiple facets of A&P, allowing students to exercise their critical thinking skills through problem solving. In its first stage, students were asked to understand the physiological aspects of the liquid chemical solutions. In the second stage, the students are asked to make the correlation between the malpractice of improper liquid chemical solution use and its ethical implications (Table 2).

During my initial semesters of teaching this course, I had also noticed that many students struggled with the concepts of basic math and chemistry. A loose grasp of these topics would create a significant problem when these topics integrated with aspects of Anatomy and Physiology. I designed a particular case study for the purpose of making these three areas of study easier to understand and fun to learn, while encouraging the development of a healthy lifestyle (Table 3).

Data Sources

The final exam grades for all of the students that had completed the aforementioned A&P courses were assessed. The same final exam, which included 78 multiple choice questions, was administered to both the PBL-enhanced sections as well as to non-PBL sections, or control group. The final exam was cumulative, which included 25 common questions (identical across all sections of introductory A&P in the biology department) and 53 questions that I had chosen at random. Grades D or above are required to pass for a majority of the courses in BCC. Grades D or above were calculated as the passing grades for the course. However, grades of a C+ or greater were calculated among the test results from all studied groups separately, as these are the minimum grades required for successfully passing the courses in Allied Health major programs, i.e. Nursing, Radiological Technology and Nuclear Medicine. The pre- and post-test results were also compared for the PBL-enhanced sections for the purpose of measuring the students' progress in the application of the topics presented through the aforementioned PBL case studies.

Analyses

Both descriptive and inferential statistics were utilized in the analysis of the data. Means, standard deviations, and t-tests for both the PBL-enhanced sections and the control sections were used ($p \leq 0.05$) to determine the statistical significance in comparing the final grades and pass rates of the students that participated in the study.

Results

The results showed that the pass rate for students enrolled in the PBL-enhanced sections of A&P was consistently higher than for students enrolled in the non-PBL, or control sections, for each semester. To assess the statistical significance in the degree of consistency of these results over time, the mathematical average of the measured parameters and their standard deviation were calculated (Fig. 1). For all six semesters, the average rate of passing students enrolled in the PBL-enhanced sections of A&P I was 93.3%, while only 87.2% of the students enrolled in the non-PBL, or control, sections received passing grades, marking a 6.1% improvement. A relatively low standard deviation in the average passing grade for the students enrolled across both sections (0.64% for PBL-enhanced sections and 0.72% for control sections) supported the finding that the consistency seen in the passing grades of the two courses, respectively, was significant.

For the purpose of confirming the importance of the 6.1% improvement witnessed in the pass rate of the students who were enrolled in the PBL-enhanced sections, the results were subjected to a t-test analysis. The results, evidenced by the two-tail p-value (by the two-sample unequal variance method) of $p = 0.0001$, further confirmed the statistical significance of the study's findings (i.e., it is less than 0.05) (Fig.1)..

Final grades of C+ or greater are considered passing marks in this course for Allied Health major programs. Students enrolled in the PBL-enhanced sections demonstrated a greater propensity in achieving grades of C+ or above in comparison to the students enrolled in the control sections for all of the studied semesters ($p = 0.01$) (Fig.2).

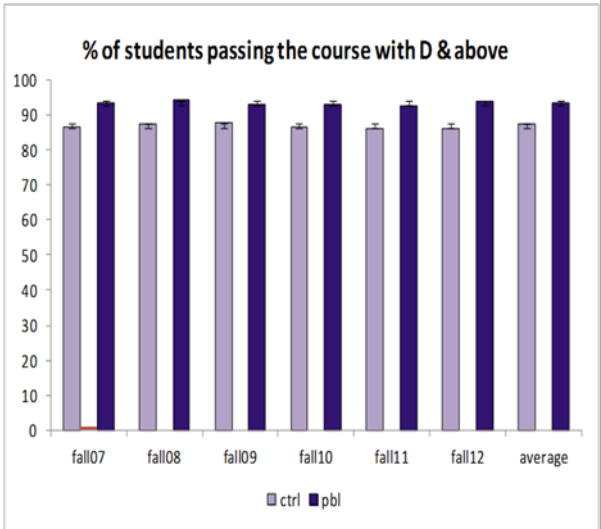


Figure 1. Consistency in improvement of passing rate for PBL-enhanced sections of introductory A&P over control sections (through six semesters). The average passing rate is 93.3% (SD ±0.64%) for PBL-enhanced sections while it is 87.2% (SD ±0.72%) for control sections, leading to an increase of 6.1% in overall performance. This improvement was found to be statistically significant according to t-test analysis (two-tail $p = 0.0001$).

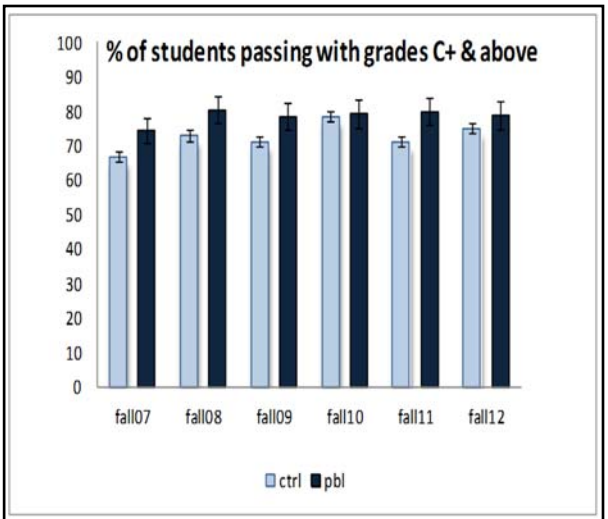


Figure 2. Comparison of student success rates between PBL and the control groups. Improvement in the rate of grades C+ and above as a result of using PBL case studies. The increase in PBL-enhanced sections was tested statistically significant ($p = 0.01$) for all six semesters.

The topics that had previously been identified as being areas of difficulty for the students through formal analysis of correlated data from previous semesters of introductory A&P course (as explained before) include, but are not limited to, the chemistry of life, homeostasis, cell/ membrane transport/solutions, nervous system/ action potential and integumentary system/ tissues. For the purpose of assessing the impact that the use of the PBL case studies had upon the students' performance in the previously noted areas of difficulty, the respective results of the pre- and post-test for the PBL-enhanced sections were compared. A comparison of these results highlighted the improvements which the students who had enrolled in the PBL-enhanced sections A&P experienced, displaying a greater propensity for mastery of the previously identified difficult concepts across all six semesters. Participation in the PBL-enhanced sections of A&P led to improved test scores of nearly ten points for all enrolled students in all of the subject areas tested (Table 4).

Conclusion

Critical and analytical thinking is a major challenge for a majority of the students enrolled in A&P courses. As educators, it is important for us to constantly improve the effectiveness of our teaching by applying different teaching methods to topics in which students experience the greatest difficulty and the poorest performance. The reasoning for such poor performance could be a lack of proper preparation during the earlier stages of a student's education. In any event, addressing the possible deficits that a student may arrive with for the purpose of better preparing them for the future is one of the main goals for us as educators. It is also our responsibility to exhaust every effort so that we may try to overcome these deficits in time.

The importance for educators to be successful in an area such as this is inherent in the careers that A&P students wish to pursue; such as nursing, nuclear medicine, radiology, and other allied health programs, where critical thinking skills and problem-solving abilities are a requirement of the profession. A lack in understanding over anatomical or physiological subject matter, or difficulty experienced in translating classroom theory to real-world application, could pose a significant threat to the allied health professions (i.e. nursing) as adverse patient outcomes have the potential to irreparably tarnish public perception[6]. Through allowing students to relate the concepts that are discussed in the classroom to the real-world consequences faced by allied health professionals in everyday life, the use of PBL

Subject areas used in PBL activities	Pre-test; Mean±SE	Post-test; Mean±SE	p value (p≤0.05)
Chemistry of life	66.68±1.41	75.51±0.92	6.107E-06
Homeostasis	70.84±0.58	78.49±0.67	9.78E-04
Cell/Membrane transport/Solutions	63.47±1.09	73.61±1.18	1.5E-04
Integumentary system/tissues	73.52±1.30	84.04±1.16	2.10E-05
Nervous system	68.44±0.90	76.7±0.96	1.61E-05

Table 4: Students' improvement in mastery of difficult A&P concepts resulting from use of PBL case studies, as measured by pre- and the post-tests. Results shown are mean ± standard error and p values to test for statistical significance (t test analysis, two tail)

case studies in the A&P classroom can better prepare our students for success in their careers.

This study identified that the use of PBL case studies in introductory A&P courses increases the students' performance and increases their knowledge of topics previously identified as difficult through their application in real-world scenarios. The results demonstrated that the use of PBL case studies in A&P classrooms increased the pass rate of those students by 6.1% on average, over the course of the six-semester period studied, versus the pass rate of those students who received traditional lectures. The stability in the pass rate of the students who were enrolled in the PBL-enhanced sections of A&P signified that the use of PBL case studies in A&P classrooms can result in performance increases over all of the general school's population.

The results indicated that the use of PBL case studies produced both higher passing grades of D or above, as is required by a majority of the Bronx Community College's classrooms, and grades of C+ or above as is required by Allied Health major programs. Significant increases in both the pre-and post-test results of the previously identified areas of difficulty for students enrolled in the PBL-enhanced sections of A&P were recorded across all studied semesters. These findings support the available literature regarding the positive impact that the use of PBL has on student learning; particularly in making connections between practice and theory and the retention of information through analysis and review[6,12-15].

Problem-based learning improves student engagement by relating content taught in the classroom to real world application. These skills enable students to be lifelong learners[16]. One informal observation that was able to be taken away from this study was to visibly see an increase in the students' interest and engagement in the course material from those who were enrolled in the PBL-enhanced sections of A&P. The application of biological concepts to authentic, real-world situations is a natural part of the Problem Based Learning teaching method. The positive impact that working on case scenarios through the use of small groups for student education is a hypothesis that was originally supported by Barrows[8], and later by others[16-19]. The teamwork that the PBL method promotes through small group discussions create an excellent educational setting for the students and ultimately leads them to build the critical, analytical thinking, and problem-solving skills they need to be successful in their professions in the allied health fields.

The PBL case studies were designed into two separate stages so that the students who enrolled in the PBL-enhanced sections of A&P would have the opportunity to build upon their knowledge from the previous stages. This two-tiered approach not only provided the opportunity to introduce some of the more difficult concepts that the students had previously experienced problems with, but also was able to integrate concepts that provided for the real-world application of the material, such as clinical problems, the topic of pharmacology and the issues associated with malpractice in today's allied health workplace. During the PBL exercises, students were asked to use their textbooks to work on the problems presented in the case studies. It was noticed that this significantly improved the academic outcomes for many of the students enrolled in the PBL-enhanced course.

The increase in overall student performance and course pass rates that directly resulted from the use of the PBL method in introductory A&P courses is statistically significant and demonstrably consistent. The students who were enrolled in the PBL-enhanced sections of A&P also demonstrated an increase in their performance when tested on topics where they had previously experienced difficulty (i.e. chemistry of life, cell/membrane transport/solutions, etc.). When coupled with the advancements made by the students in establishing connections across various topics in multiple disciplines and the improvements made in the ease with which they were able to apply the content learned in their classrooms to specific, real-world problems through their use of critical and analytical thinking skills and problem-solving abilities, the overall increase in the pass rate of the PBL-enhanced introductory A&P section and the positive impact on student performance which the use of the PBL method in the introductory section of A&P was found to have suggests that the students who benefit from the use of the PBL method will ultimately be better prepared for their careers in the allied health fields and will be better equipped to tackle the problems that tomorrow's allied health professionals will face.

In time, I imagine that my development of PBL case studies will evolve from the use of discussions originating on Blackboard to the linking of case studies with interactive games and questions, possibly creating a self-directed web-based forum for case studies to be used in collaboration with small group discussion and in-class group work[20]. Recent literature suggests that a combination of traditional style teaching, web-based study and classroom discussion will be the next advancement in teaching A&P in allied health courses[5,14,21,22].

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Molecular Analysis and Structural Prediction of Glial Fibrillary Acidic Protein in Alexander Disease

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Abstract

Alexander disease (AxD) is one of a large and diverse number of neurodegenerative diseases recognized as “protein conformational diseases”. It is caused by a point mutation in the gene encoding a cytoskeletal protein glial fibrillary acidic protein (GFAP). Microscopically, it is characterized by the copious presence in astrocytes of protein clumps termed Rosenthal fibers (RFs). Understanding GFAP misfolding and aggregation in the CNS would shed light on how the cell controls its normal protein balance in all “protein conformational diseases”, which are currently without cure. In our study, we acquired autopsy brain tissue from a AxD patient with R239C mutation, the most common mutation identified in AxD. By molecular biology approach, we demonstrated that mRNA from both the normal and mutant alleles in this patient was equally available for GFAP synthesis. To further explore the assembly defects of R239C GFAP demonstrated by the stable human astrocyte line of AxD, we investigated protein surface features and probabilities of coiled-coil formation utilizing different online protein databases. We found that that R239C GFAP has a higher solvent solubility and a lower probability of forming coiled-coil heptad repeat motif compared with wild-type GFAP. Our structure-based predictions are in good concordance with experimental findings from different cell types and mice model of AxD. Both suggest that altered oligomerization kinetics of R239C GFAP might contribute to more intermediate and aggregated forms of GFAP in RFs. To the best of our knowledge this is the first study that incorporates allelic expression profile in conjunction with sequence based secondary structure prediction to gain an insight into the effect of R239C mutation on conformational changes of GFAP in AxD.

Keywords: Alexander disease, GFAP, Rosenthal Fibers, astrocytes, allelic imbalance, protein structure prediction

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Introduction

Alexander disease (AxD) is an autosomal dominant neurogenetic disorder caused by mutations in the gene encoding glial fibrillary acidic protein (GFAP)[1,2]. Common features of AxD include macrocephaly, psychomotor regression, intractable seizures and eventual death. Three forms (Infantile, juvenile and adult) of AxD have been described with variable clinical severity. Notably, AxD patients with mutant GFAP protein containing an altered arginine

residue (Arg239 or R239) tend to present with early onset and more rapid progression of disease[3]. Regardless of a wide spectrum of GFAP mutations and the clinical symptoms displayed, all identified AxD patients are unified by the presence in astrocytes of prominent Rosenthal fibers (RFs)-protein aggregates that contain glial fibrillary acidic protein (GFAP), small stress proteins, plectin and ubiquitin[4-6]. A deeper understanding of RFs requires an appreciation of the filamentous organization of both wild-type and mutant GFAP in human astrocytes. In our study, we applied a combination of cellular, molecular techniques and bioinformatic tools to explore the expression, structure and function of wild-type GFAP and R239C GFAP.

Materials and Methods

DNA cloning enzymes were purchased from New England Biolabs. TA cloning kit was from Fisher (K451022). Cell culture medium and supplements were from Sigma. Superscript II reverse transcription kit was from Gibco. RNA isolation from human brain tissue, amplification of retroviral expression vectors (WT-GFAP-IRES-eGFP and RC-GFAP-IRES-eGFP) in gp293 cells with the help of VSVG protein; Confocal microscopy and cell line selection with Fluorescence Activated Cell Sorting (FACS) were performed as previously described[7].

Brain Tissue

R239C AxD case was diagnosed based on histopathologic examination and confirmed by molecular genetic analysis for GFAP mutation. Control tissue was frozen CNS tissue from a child (no neurological disorders) with watched gender, age and postmortem interval (male, aged 20 months, 18-hour postmortem interval).

Plasmids and expression Constructs

U251 human astrocytoma cells were infected with retroviral construct pQCXIX-IRES-eGFP (modified from Clontech's pQCXIX) containing cDNA encoding wild-type (WT) or arg239cys (R239C) GFAP inserted between the BamHI and *Eco*RI sites. Plasmids Topo-2.1 and pcDNA3.1/ myc-His C were kindly provided by Dr. Ron Lim (Columbia University, New York).

Bioinformatics

The NCBI website was used to retrieve the FAFTA format of wild type human GFAP; the Sequence Manipulation Suite (a JavaScript program for analyzing and formatting protein and DNA sequences) to obtain the protein format for both wild-type and R239C GFAP protein via www.bioinformatics.org/sms/. Different web servers were employed to determine whether the sequences will be considered a coiled coil by three coiled coil prediction programs:

Coils program: http://www.ch.embnet.org/software/COILS_form.html

Paircoil program: http://groups.csail.mit.edu/cb/paircoil

Marcoil program: http://www.isrec.isb-sib.ch/webmarcoil/webmarcoilC1.html

The performance of Coils, Paircoil and Marcoil was compared and interpreted as sensitivity, specificity. The sensitivity was defined as true positive/(true positive + false negative) and shows the proportion

of sequences of coiled coils that were correctly predicted, the specificity was defined as 1- false positive rate = True negative/ (True negative + false positive) and represents the proportion of non-coiled coil sequences that will not be predicted. Reliability was defined as True positive / (True positive + False Positive).

Results

Nucleotide and amino acid sequence of WT and R239C GFAP protein

We obtained the human wild-type (WT) GFAP data base from the NCBI website. The FASTA format gave us the complete 3,097 base pairs. We removed the first 66 nucleotides (highlight in red), which left 3,031 nucleotides, to display the start codon (ATG) for the GFAP sequence (Fig. 1a). Next, we determined the nucleotide sequence of R239C GFAP, the most frequent mutation among AxD patients. After manually counting to 717 nucleotides (239*3=717, R239C) in a word doc, we compared the possible codon sequence from a genetic code table for both arginine (239 R) and cysteine. We reasoned that the first C (cytosine) within the triple nucleotides (from 715 to 717) must be replaced by T (thymine) to code for amino acid cysteine (Fig. 1b). Lastly, we used The Sequence Manipulation Suite (http://www.bioinformatics.org/sms/) for a cDNA-derived amino acid sequence (Fig.1a) of WT GFAP. We removed everything past the first (*) symbol because it indicates a stop codon (length=432aa, Fig. 1c).

Domain analysis of GFAP protein

GFAP is a type III Intermediate filament (IF) protein. All IF proteins consist of three domains: the central highly conserved, extended a-helical rod domain flanked by non-helical head (N-terminal) and tail (C-terminal) domains of varying lengths. The rod domain is

Fig.1 Nucleotide and amino acid sequence of WT and R239C GFAP

Fig.1a shows the first 85 nucleotide sequence after manually removing the first 66 nucleotides (in red) for WT GFAP on a word doc

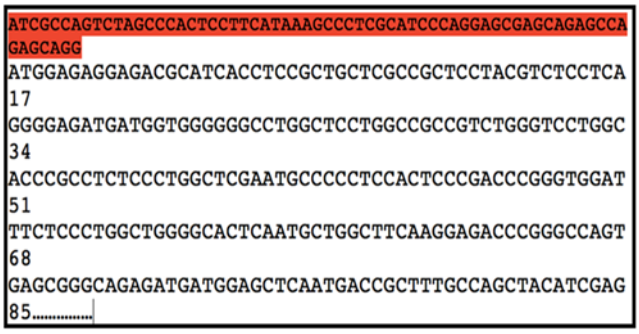
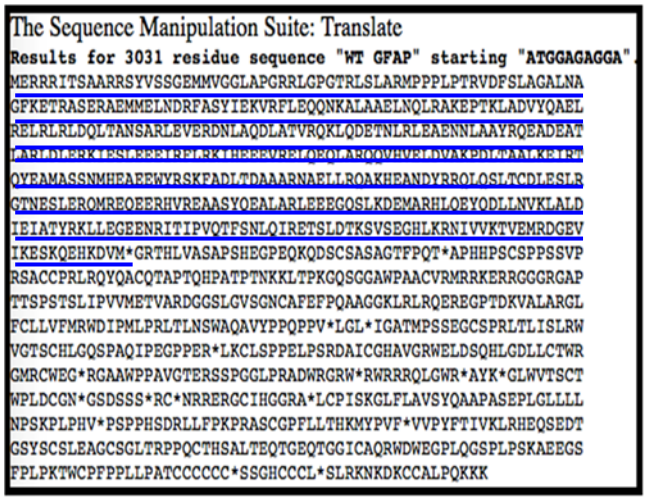


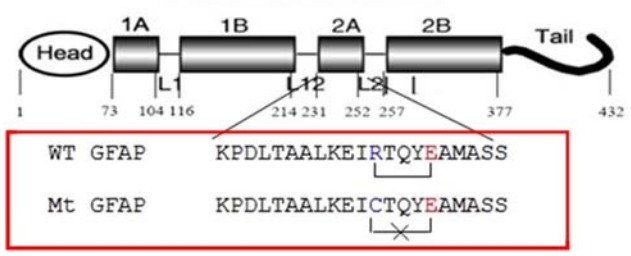
Fig.1b highlights the nucleotide, C, replace by T in position 715

Fig.1c shows the amino acid sequence of wild-type GFAP (underlined in blue)



essential for polymerization and has four coil subdomains, 1A, 1B, 2A and 2B interpersed with flexible linker segments[8]. Recent application of high-resolution atomic force microscopy has generally confirmed the previous hypothesis that the formation of a multi-stranded architecture of IF by distinctly aligned coiled coils (two or more alpha helices are intertwined into a stable rope-like structure) can be evaluated through several hierarchical levels of increasing order and complexity[8]. The primary structure of human GFAP (Swiss rot, accession P14136) shown in Fig. 2. R239C mutation is located in the segment 2A, exon 4 region of the rod domain.

Fig. 2 The schematic structure of wild-type man GFAP protein



It is composed of four supposed helical coiled-coil rod domains [segments 1A (73-104aa), 1B(116-214aa), 2A (231-252aa) and 2B (257-377aa); linker L1 , L12 and L2], a randomly coiled head (1-72aa) and a tail region: 378-432aa . R239C substitution occurs in the exon 4 within the 2A rod domain (www.pdb.org/pdb/protein/P14136#), leading to a loss of the potential intra-helical salt bridge between amino acid 239 R and amino acid 243 E (the zoom window of domain 2A in red).

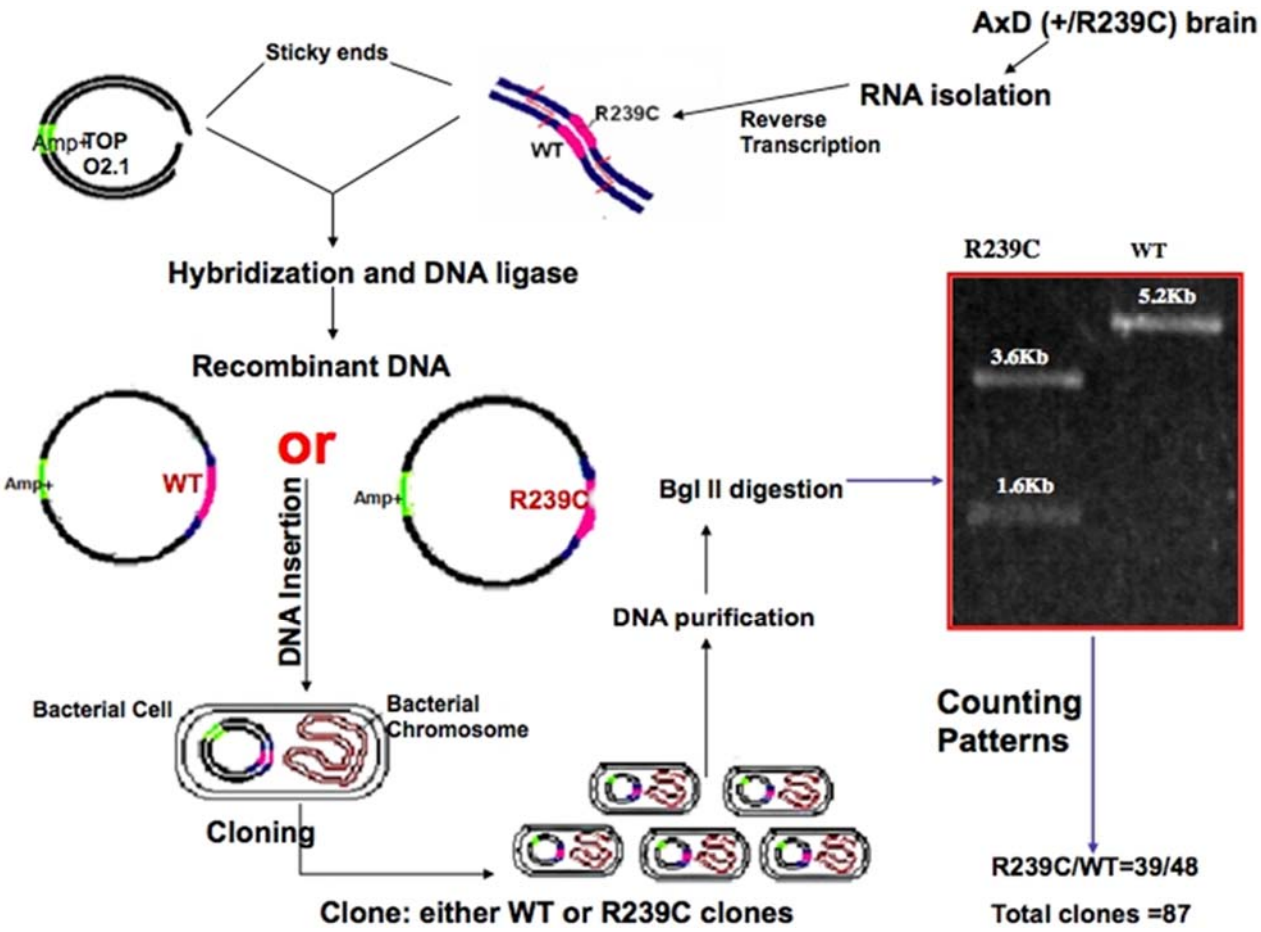
Quantification of GFAP transcripts in R239C AxD

Nearly all AxD patients are heterozygous for the respective mutation (http://www.waisman.wisc.edu/alexander-disease/diagram.html). Both mutated and wild-type proteins are assumed to be co-dominantly expressed from the corresponding alleles. Yet, a higher or lower expression of the one particular allelic transcripts and proteins resulting in a deviation from the expected 1-to-1 ratio, known as allelic imbalance, could well be quite significant for disease expression in AxD. To address this question we measured the relative abundance of mutated and wild-type *GFAP* mRNA in an AxD patient carrying R239C mutation. Sequence comparison at amino acid 239 (Fig.1b, **C**GC->**T**GC; Arg->Cys) revealed a new target site for the restriction enzyme Bgl II. Bgl II recognizes and cuts the R239C GFAP nucleotide sequence at A^GATC**T** site, but fails to do so on the wild-type GFAP nucleotide sequence AGATC**C**. Thus a cDNA fragment spanning the mutation site was amplified from RNA isolated from the frozen neocortex and white matter of this patient (reverse transcriptase-PCR). The 1.3kb PCR product was subcloned into Topo 2.1 vector and analyzed by Bgl II digestion. Fig. 3 shows equal transcription of wild-type and R239C mutant *GFAP* allele with a simple RFLP (restriction fragment length polymorphism) assay.

Generation of stable human astrocytic cell via retroviral vectors expressing the same levels of WT and R239C GFAP mRNA

Growing evidence suggests that in addition to equal transcription, co-transfection of equal amounts of plasmids encoding WT and other type of GFAP mutations of AxD in plectin-null fibroblasts and IF-free *SW13* cellsresulted in equal amounts of the respective GFAP proteins being produced[5,6,9-11]. Thus, we designed bicistronic vectors expressing either WT or R239C GFAP mRNA fused to Green Fluorescent Protein (GFP). These vectors (modified from Clontech's pQCXIX)) contain IRES, the encephalomyocarditis virus, which allows efficient translation of polycistronic mRNA[12]. In brief, U251 human astrocytoma cells were infected with retroviral construct pQCXIX-IRES-eGFP containing cDNA

Fig.3 Equal expression of mRNA from R239C and WT GFAP alleles



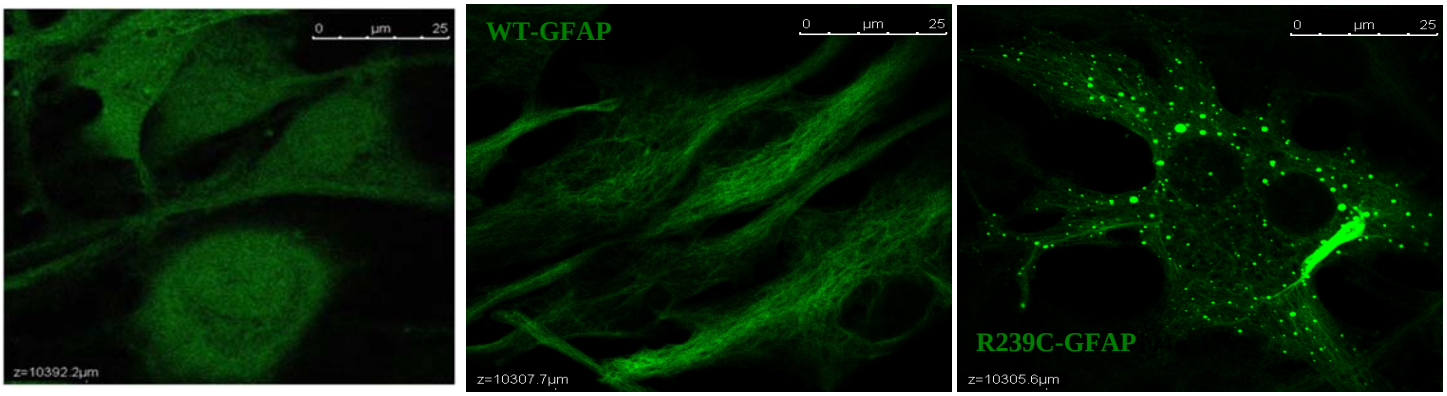
We directly examined the mRNA expression profiles of the WT and R239C GFAP genes from an AxD patient expressing the R239C mutation using an RT/PCR-RFLP analysis**.** After Bgl II cleavage, WT allele gives a 5.2kb product, while R238C allele gives two bands at 3.6 kb and 1.6 kb. Of the 87 clones analyzed, 39 were R239C mutant, 48 were WT, suggesting an equal ratio of the two transcripts.

encoding WT or R239C *GFAP* inserted between the BamHI and *Eco*RI sites. FACS was then used to select cells expressing same levels of GFP, a reporter gene in our retroviral construct stably integrated into the astrocyte genome. GFAP and eGFP expression levels from this construct are directly proportional; cells expressing high levels of GFP contain high levels of GFAP, and vice versa[7]**.** Fig.4 shows WT-GFAP frequently displays a typical, filamentous network in a low but stable expression level, but the same levels of R239C GFAP are sufficient to induce aggregation in human astrocyte cell line.

Bioinformatic predictions in the secondary structure of GFAP

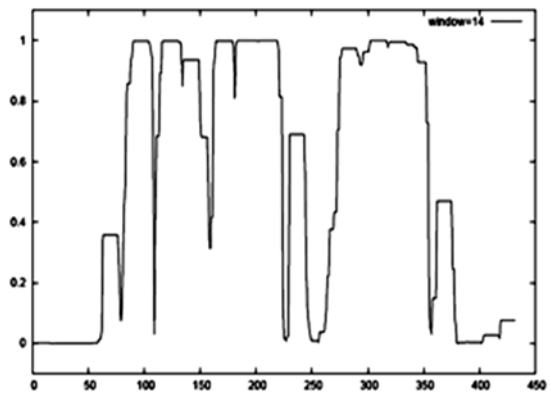
During the optimization process of GFP+ stable cell lines, we observed that with increasing levels of GFAP, R239C GFAP aggregates more rapidly than WT GFAP in these astrocytes. The increased propensity for R239C GFAP to form inclusions was also reported in studies using transient expression assays and mice models of AxD[9,13,14], strongly suggesting that the structure of the mutant GFAP is severely perturbed. However, due to the lack of adapted conditions for IF crystallization and the insolubility of GFAP

Fig. 4. Generation of stable human astrocyte lines using retroviral vectors and Confocal imaging

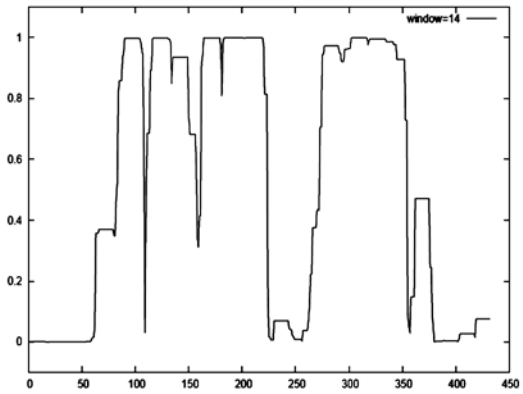


A majority of R239C astrocytes exhibits irregularly shaped processes and perinuclear aggregates; whereas WT-GFAP astrocytes formed well-organized GFAP filament bundles at similar levels.

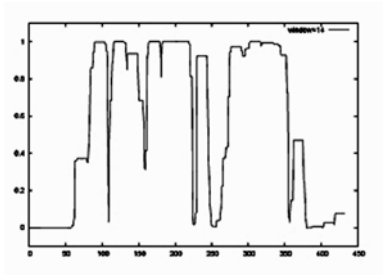
Fig. 5. Predictions of R239C substitution on GFAP coiled coil formation



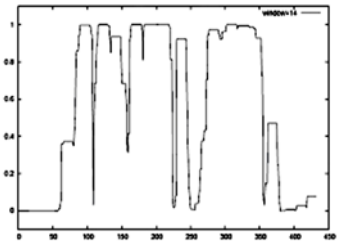
Coils output for WT GFAP



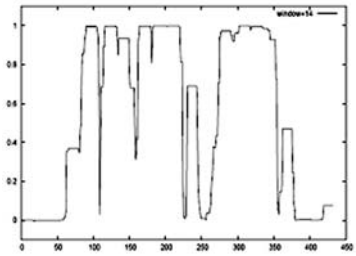
Coils output for R239C GFAP



R79H GFAP



R239H GFAP



R416W GFAP

The coiled coil structures of full length WT, R239C, R79H, R2399H and R416W mutant GFAP protein were predicted using unweighted Coils program with a MTIDK matrice. Arrow: a predicted coiled coil structure in rod domain 2A (220-255aa with14 residues as sliding window size). X axis: the order of amino acid, Y axis: the probability cutoff value. Red arrow showed a loss of coiled coil in R239C GFAP, which is present in WT (dark arrow) and all other mutant GFAP (R79H, R239H and R416W).

proteins, the accurate determination of GFAP three-dimensional structures using x -ray diffraction (XRD) or nuclear magnetic resonance (NMR) spectroscopic techniques is not available at present. Alternatively, we attempted a simplified approach by predicting the secondary structures of the WT and R239C GFAP protein.

Table 1 Predicted solvent accessibility composition (core/surface ratio)

	Accessible type	Buried	Exposed	Intermed
WT	% in protein	28.47%	62.05%	9.03%
R239C	% in protein	26.85%	63.89%	9.26%

The prediction of GFAP secondary structure was done via predict protein algorithm (https://predictprotein.org/). First, the alpha helix within domain 2A is stabilized by an intra-chain ionic interaction, or salt-bridge, between arginine (R) 239 and glutamate (E) 243 within domain 2A. R239C substitution disrupts this interaction potentially leading to the instability of the alpha helix itself, and consequently the coiled coil motif between alpha helices (Fig.2, red window). Next, we found that R239C substitution changed the surface exposure of the folded GFAP protein from 62.05% to 63.89% based on predictions of WIWS[15] (table 1). We further explored the effects of R239C mutation on the formation of 2A coiled coil via COILS. Among several hotspot mutations for AxD, R79H locates in GFAP protein head domain, R239C and R239H reside in the rod domain 2A, and R416W mutation resides in tail domain of GFAP protein. The unweighted Coils program with a fixed window size 14 and MTIDK matrice of GFAP protein predicts a coiled coil structure in the rod domain 2A in WT GFAP .The R239C mutation, however, resulted in a loss of this coiled coil structure with the predicted probability decreased from approximately 0.7 at amino acid 239 to approximately 0.1 (Fig.5 top), while the other three mutations showed no effects (Fig.5 bottom).

Discussion

Given RFs with elevated GFAP protein are the primary pathological feature of all AxD patients, we set out to determine whether the wild-type and mutant transcripts in AxD patient are represented equally or whether there is a skewed distribution of one with respect to the other. An unequal distribution might argue for differences in the stability of the wild-type and mutant transcripts. A RT-PCR followed by RFLP performed on post-mortem brain tissues of a AxD patient bearing R239C revealed equal transcription of wild-type and R239C *GFAP* allele. Our results suggest that elevated GFAP protein in RFs might be driven by post-transcriptional or post-translational mechanisms (e.g. promoting GFAP aggregation or stability by an increased GFAP translation and/or a decreased GFAP degradation).

To observe the phenotype of R239C GFAP in human astrocytes directly, we created a stable cell line. We found that, in contrast to a typical filamentous phenotype of WT GFAP, very low levels of R239C GFAP transcripts are sufficient to produce RFs-resembling aggregates. Additional studies in these cells and mice model of AxD not only confirmed our initial findings that both alleles are equally transcribed, but further elucidated that a combination of complex post-transcriptional events, which include altered polymerization of mutant GFAP, SAPK/JNK activation, and an impaired proteasome and activated autophagy, might synergistically contribute to the exacerbation of GFAP aggregation in AxD[6,11,16,17].

Considering most GFAP mutations in AxD have been found in the 1A, 2A and 2B segments of the rod domain, it is likely that the mutations in the rod domain impair the micromechanical properties by compromising different phases of GFAP filament assembly, such as elongation, lateral/self association, fibril nucleation, oligomerization, filamentous bundle packing, etc. However, an accurate determination of the 3D structure of GFAP using XRD and NMR has so far been unsuccessful. Hence, we employed a number of web-based bioinformatic programs to study the sequence based secondary structure of R239C GFAP. Our analysis indicates an amino acid substitution R239C increases the accessibility to the solvent and decreases the stability and probability to adopt a coiled-coil conformation. GFAP filaments are dynamic structures, maintained by a rapid and continual exchange of subunits between the small pool of kinetically active unassembled GFAP and a larger pool of assembled (polymerized) GFAP filaments. The unassembled GFAP, mostly dimers and tetramers, are soluble in low ionic strength buffer, for example, 1% Triton[18,19]. The increased surface accessibility of R239C GFAP is consistent with a significant higher proportion of Triton-soluble fraction (monomers, tetramers and short filaments do not sediment at 15,000 × *g*) in astrocytes stably expressing R239C GFAP[5]. Higher levels of unassembled/misfolded GFAP and a potential loss of coiled coil in domain 2A-2L of R239C GFAP might confer a change of oligomerization kinetics of GFAPs, leading to more intermediate and aggregated forms of GFAP[20,21]. Future studies with mutation-specific antibodies and proteomics/mass spec aiming to identify all proteins present in the aggregates will provide more definitive clues to their formation and to their biological effects in AxD.

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The Use of Audacity and Praat to Record and Analyze Animal Vocalizations

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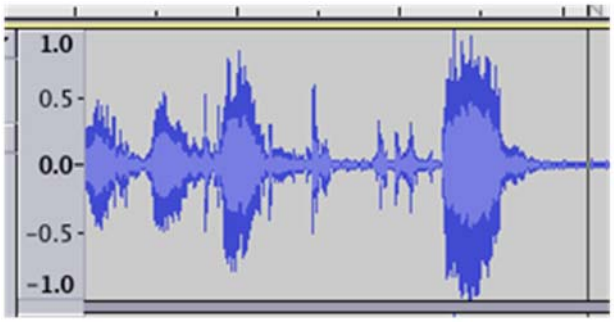
Abstract

The research project of the Intrepid Sea, Air, and Space Museum Navigators was three-fold and was based on: 1. direct observation, recording, and analysis of sea lion vocalizations at the Bronx Zoo, 2. the recording and analysis of animal vocalizations from an on-line sound library, and 3. conducting our own individual research on various animals we are interested in. Intrepid Navigators have been recording animals both "in the wild" at the Bronx Zoo and from the Cornell University Macaulay Library of Sound Recordings. This is in an effort to learn more about animal vocalizations. Are there patterns associated with the sounds? Are they different for males, females, and different age groups? Can we detect individual differences? Can we associate these vocalizations with specific behaviors such as territorialism or calling a pup? Through using the two free downloadable programs Audacity and Praat waveforms are recorded and then converted to spectrograms for further analysis. The analysis consists of recording: 1. Number of times animals vocalize 2. Type of vocalization, such as a series of barks, growls, or trills, 3. Time duration of vocalization 4. Association with a particular behavior 5. Number of formants (resonating waves) associated with a subset of the vocalizations. 6. Range of frequencies 7. Differences that we note within species. This trip spurred us on to conduct further research on learning about animal behavior through vocalization. We recorded and analyzed sounds from the sound library, and did additional peer-reviewed paper research.

Introduction

We visited the Bronx Zoo on January 3, 2016 and recorded vocalizations of the sea lions present there. There were seven sea lions, two males, one large adult and a year old juvenile. There were five females, one large and four juveniles. Interestingly, two pups were born there over the summer, but they recently died of unknown causes. We recorded the sea lions both visually with phone cameras, and through audio recordings using the free downloadable program Audacity. (See Figure 1 for a picture of a waveform)

Figure 1. A waveform of sea lion barks using Audacity on January 3, 2016



We observed the waveforms and counted (mostly the large male) how many vocalization he made in a 37-minute time period, how long each vocalization was, how many sub-vocalization there were---(utterances in a bark, for example) and the duration of each vocalization. The average frequency range was noted, and the number of formants in a typical "bark" was noted, as well as the frequencies of these formants. The data is summarized in Table 1.

Analysis of the behavior and vocalizations of the sea lions on January 3, 2016

The first sea lion was most likely a female due to its behavior and vocalizations. It used growls, an indicator of a female, to interact with both the male and the juvenile. In addition, the average vocalization was 0.73 s, which was more dominant than that of the third sea lion, but less compared to the second subject. Moreover, the frequency (in hertz) compared to the other vocalization was about the same as in the average vocalization- less than the second sea lion (male), but greater than the third (juvenile). This shows us that the female might have less authority than the male, but more than the juvenile. Furthermore, the female did not engage in a colossal number of vocalizations compared to the male. Based on Table 1, it can be seen that the first and third sea lions have about the same number of vocalizations, and it can be inferred that they engaged in "conversation." According to Ndez-Juricic *et al*[1]. the juvenile may have been interacting with his mother to search for her or to alert her that he is hungry. This is plausible as the recording that was analyzed was taken near their feeding time.

The second sea lion was most likely a male due to the nature and quantity of his vocalizations. This hypothesis was confirmed by the trainers. The male sea lion had 17 vocalizations, almost twice that

Table 1. Vocalizations of a Sea lion at the Bronx Zoo on 1/3/16

	Sea lion 1	**Sea lion 2**	**Sea lion 3**
# of vocalizations	9	17	8
Type of vocalization	Barks/Growl	Long Growl/High Pitched Call	Bleat
Range of duration of vocalization	0.47-1.48s	1.04-1.71s	0.47-0.97s
Average Vocalization Duration	0.73s	1.19s	0.71s
Mean Frequency (Hz)	2235.45	2366.95	2234.925
Analysis	Female, interacts with the male (SL#2) through growls, also interacts with pup (SL #3), frequency less than male, but more than pup	Dominant male (territorial claim), extremely vocal, interacting with female (Sea Lion #1), highest vocalization, long vocalizations may indicate hunger or longing for food	Juvenile, average vocalization duration shortest, lowest MF, but close to the female's, interacts with female (presumably mother)

of the first sea lion. In addition, he produced growls and high-pitched calls, both of which are usually emitted by the males. The average duration of the second sea lion was 1.19 s, substantially longer than the average of the first. The longer the duration, the greater the indication of dominance, according to Ndez-Juricic *et al.*[1]. The second sea lion was extremely vocal and appeared to be exchanging a number of vocalizations with the female (first sea lion). Furthermore, it was observed that when the trainers arrived, the sea lion was swimming and jumped onto land. As the trainers approached the gate, the sea lion began to jump and produced high-pitched calls, indicating excitement as well as hunger.

Ndez-Juricic[1] note that California sea lions, also known as *Zalophus californianus,* make various vocalizations, such as barks, whimpers, honkings, growls, grunts, and clicking sounds. Barks are used by male California sea lions when establishing territories. A female sea lion emits loud trumpeting vocalizations, which elicits a bleating response from the pup. High-pitched sounds are emitted by males to assert his dominance over other males. In addition, the males call the females through growls, which is considered their mating call. Grunts are used by the female sea lions to settle altercations between them. Furthermore, males emitting clicking sounds can be used to indicate annoyance and often predict a fight.

Figure 2. Formants of sea lion barks on an Audacity spectrogram (approximately 8 per bark)

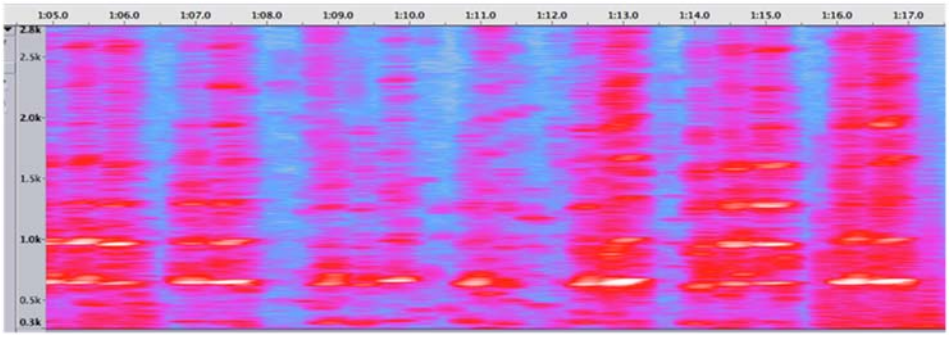
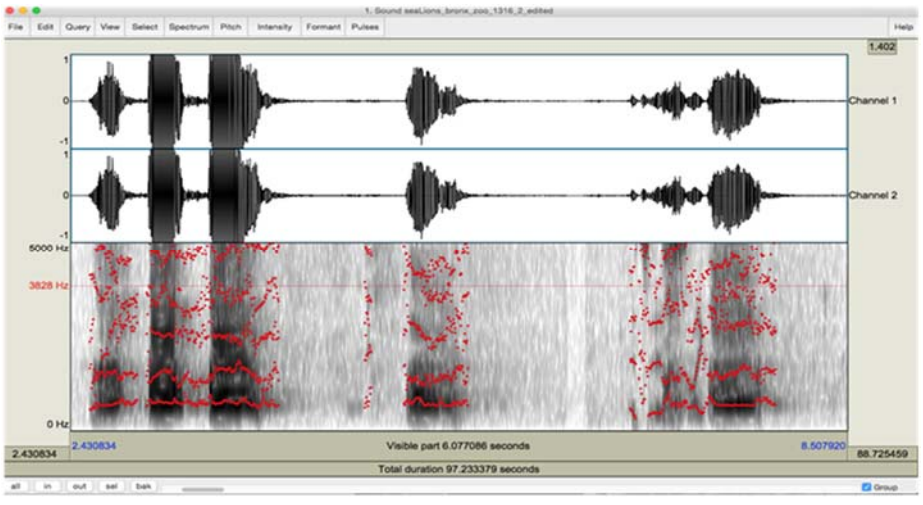


Figure 3. Incorporating a waveform into Praat to visualize formants



Based on the data collected by the researchers, the dominant males have been shown to have longer vocalization durations, higher mean frequency of the vocalizations, and a greater number of vocalizations (Figs. 2, 3).

The male was the most vocal and he appeared to be telling the world that this was his domain. All the animals were more active just before and after the trainers came to bring them food. Just as we were leaving, one of the females became more vocal and started barking, but we had put our recording devices away. From past visits, we have heard growls and bleating type voices. We did record a few loud breaths that they made when surfacing, as they spend quite a bit of time swimming underwater. Other behaviors we observed were drying themselves on a rock, porpoising, (swimming in a leaping fashion) and activities on cue from a trainer, such as jumping.

Trainers use sea lions not only for shows but to learn about how animals learn—or, animal cognition. Since the Marine Mammal Protection Act was passed in 1972, it is illegal to kill marine mammals. If they are stranded, or "rescued" they must be kept in captivity, if at all possible. While captive, these animals must receive enrichment, often in the form of training. The moves or behaviors that an animal such as a sea lion or dolphin learns can then be exploited and used in a "show". But, how do we know that animals have "learned" something—say, are able to tell one type of picture or a symbol from another? This is where the training comes in. The trainer will give an animal a fish reward if they choose the "correct" scenario, or exhibit the desired behavior. For example, it has been found through training that sea lions can learn how to keep a beat, and are thus able to distinguish one repetitive rhythm from another. Since the animals cannot "talk" to us, at least in a way that we understand, the training becomes a way to learn about animal cognition (Biolsi, 2016, personal communication).

Macaulay Library of Sound recordings, research articles, and Analysis

Something we felt that would be expedient to conducting this research project was to research and record our own animals using the Cornell University Macaulay Library of Sound (MLS). We would open up Audacity and the MLS sound recording (each has its own accession number) and press "play". In this way, we added an "online" component to our research. We were able to hear animals that we would not normally have access to—sometimes not even in a zoo. The animals make a wide range of sounds as well. One observation is that the sounds of the less aggressive animals had a pattern. Sometimes the sounds made by the aggressive creatures, such as trying to scare off prey, were more random and sporadic. Furthermore, the more aggressive/ sporadic sounds came from when in contact with another animal. The calmer calls were made not with another animal very close by, but with a possible audience.

Table 2 is a table of the animals we recorded with text and observations about the animals to follow. We then found peer -reviewed articles on animals that we had recorded and were interested in. In our research, we were trying to uncover answers to the following questions:

Table 2. Animal recording from the Macaulay Sound Library#			
Common name	**Latin name**	**Sound library recording number**	**Locations**
California sea lions	*Zalophus californianus*	ML 516298	California, USA
South American Sea lion	*Otaria flavescens*	ML 29398	Peru
Antarctic Fur Seal	*Arctocephalus gazella*	ML 196637	Antarctica
Steller Seal	*Eumetopias jubatus*	ML 193402	Alaska
Brown fur seal	*Arctocephalus pusillus pusillus*	ML 148983	Namibia
Antipodean fur seal	*Arctocephalus forsteri*	ML 125016	New Zealand
Rock Hyrax	*Procavia capensis*	ML 53588	Kenya
Ring-tailed lemur	*Lemur catta*	ML 85851	Madagascar
Gorilla	*Gorilla gorilla*	ML 126333	Rwanda
Baboon	*Papio hamadryas*	ML 55969	Kenya
African Lions	*Panthera leo*	ML 53437	Namibia
White-throated bee-eater	*Merops albicollis*	ML 212689	Cameroon
Great Blue Turaco	*Corythaeola cristata*	ML 141040	Sierra Leone
Bald Eagle (A.C.)	*Haliaeetus leucocephalus*	ML 137879	Alaska, USA
Magellanic penguins	*Spheniscus magellanicus*	ML 126048	Argentina
Little /Fairy penguins	*Eudyptula minor*	ML 896	New Zealand
Snowy Owl	*Bubo scandiacus*	ML 138288	Polar Bear Pass; Bathurst Island, Canada
Opossum	*Didelphis marsupialis*	ML 29675	Peru
Great Gray Owl	*Strix nebulosa*	ML 49944	Alaska

1. What types of vocalizations do the animals make?
2. What do they use the vocalization for? (Locate a mate, establish a territory, etc.)
3. What types of data were collected in the article? (Number of vocalizations, frequency of them, time of day, etc.)

Sea lions

Since we had seen the sea lions first, we decided to record and investigate these animals first. There are a variety of recordings for various species of sea lions, including the California, Southern, and Stellar. From the MLS, and from our research, we noted that male California sea lions bark incessantly when establishing territories, Pups make a bleating mother-pup recognition call and a high-pitched alarm call. The female emits a loud trumpeting vocalization, which elicits a bleating response from her pup. Charrier and Harcourt[2], showed through recordings that Australian sea lion mothers and pups recognized each other's vocalizations. In general, consistent with our Bronx Zoo trip,

Table 3. Types of Sea lion calls and associated behaviors (Charrier and Harourt[3])			
Call Type	**Sender**	**Recipient + %**	**Behavior context**
High pitched call	MALE	Male 68.9%# Female 5.9%# Colony 5.9%	Aggressive interactions (attacks, fights)
Bark	MALE	Colony 86.5%# Male 5.3%# Female 3.7%	Territory establishments
Growl	MALE	Female 50.6%# Male 18.4%# Colony 24.1%	Male-female interactions, mating call
Mother primary call	FEMALE	Pup 78.8%# Colony 24.1%	Female-Pup interactions (separations after birth, pup development)
Grunt	FEMALE	Female 74%# Male 12%	Altercations between females
Pup primary call	PUP	Female 88.5%# Male 0.8%# Colony 10.3%	When hungry, searching for their mothers, trying to nurse/when nursing is interrupted

vocalizations include barks, growls, and grunts. During periods of nonbreeding, submissive males become more vocal than dominant males. Some observations made about the Southern sea lions (that we were then able to compare and contrast with our observations from our group Bronx Zoo trip) include the following: the Southern sea lions make barks, whimpers, honkings, growls, grunts, and clicking sounds[1]. An adult male will let out a deep guttural grunting sound that asserts his dominance to other males on the beach. Clicking sounds can be used to indicate annoyance and predictions for a fight. Table 3 depicts a summary of some of the types of vocalizations that were recorded, and possible behaviors they intimated.

Sea lions will also produce underwater vocalizations that include loud barks, whinnies, faint clicks, moans or humming sounds, chirps, belches, and growls. Underwater vocalizations seem to be used by sea lions during breeding seasons, especially males, to assert dominance and define territories. When there is a threat to a harem or a colony of sea Lions, loud trumpet sounds will be given. This is a signal to them all that there is danger lurking on land. Their instincts are to get into the water and off the land. It is complete chaos and many Sea Lions end up trampled in the confusion, especially the younger ones (Table 3).

Charrier *et al.*[3] conducted playbacks of barks of varying speeds and frequencies to Australian sea lions. They found that they responded more quickly and turned toward playbacks that were at the control speed and faster, and ignored playbacks that were slower than the control. Schusterman[4] states that sea lions make two types of vocalizations--one that occurs above water and the other which occurs below water. The sea lions' underwater click vocalizations may be related to a general arousal

phenomenon, which is related to "questioning reaction" or "orientation reflex". Click vocalizations are most likely used to convey information concerning the moods of the sea lions so they become a part of the underwater communication that animals have with one another. The authors focus on the arousal phenomenon and how it plays a part in the sea lions social behavior and rand emotional character. The researchers wanted to see how the sea lion would react when introduced to a new stimulus and collected data to this effect. So they inserted mirrors into the sea lions' environments to see that effect on their underwater vocal stimulation. Researchers decided that mirrors would be a good decision because reflected self -images produce intense interest in chimpanzees. This would be also evident after the mirror test on the sea lions.

The animals did not vocalize before or after the test periods but all immediately produced underwater clicks when initially exposed to their reflection. These clicks decreased after every test as the it seems the sea lions became accustomed to seeing their reflection. Two animals even tried to ram into mirrors while making a lot of clicking noise showing that they were not able to recognize their reflection as their own reflection and thought it was another animal. The data that was recorded in the article showed number of clicking during 30 seconds intervals in 5-minute periods. Scientists produced a graph of their data. For a control with no mirrors, the animals were paired with each other on two separate occasions and for one hour each animal was allowed to get accustomed to its surroundings for one hour. Then, the underwater vocalization of the free-swimming animal was recorded for 20 minutes and then the second animal was introduced into the tank and both animals' vocalizations were recorded for another twenty minutes. From the graph we can tell which animals are more vocal and how the animals interacted with one another. The research shows that the sea lions' investigative behavior is based on visual orientation and their underwater click vocalizations also conform to visual orientation as shown by experiments with mirrors.

Large terrestrial mammals

African lions use a variety of vocalizations, most notably the roar. It can be heard over a distance of 8 km. and serves to let other members of the pride know where they are, and as a signal to strange males to stay away. Vocalizations are used for territoriality, intimidation, social bonding, and advertising their presence. Other sounds include: growl, snarl, hiss, meow, grunt, and puff. Puffing, which sounds like a stifled sneeze, is used in friendly situations. Males begin roaring at one year - females a few months later. There is a gradation in volume with age[5].

Gorilla

Gorillas have many ways in which they communicate, both verbally and physically. Gorillas can be very loud when they are communicating. They often mix sounds with actions and that makes it clearer to researchers what is being said. One example is when a confrontation between male gorillas occurs. Adult males will not back down when they are being challenged by younger and immature gorillas. They will make very loud screaming sounds and at the same time they will beat their chest with their hands rapidly. This is a warning signal to the

younger gorillas to back off or they will be engaged in a battle. Most of the time the younger ones will retreat. Researchers have identified 20-25 different sounds that gorillas make and what they mean. They make screams, grunts, roars, growls, and even hooting like an owl at times.

Gorillas use their communication skills for a variety of things. This is one of the most important things that a mother can teach to her offspring. They use their communication to find food, to offer support or discipline, to express their own distress, for mating, and for developing social relationships within their troop. What is also very interesting is that in certain troops they may develop forms of slang as we do in our own social groups. This is fascinating as it means that their communications are often learned behaviors and not just an instinct. It can be harder though for gorillas when they move to find their own troop as those forms of communication will not be readily known by others outside of that troop. It is kind of like someone who is speaking Spanish with an Argentinean's dialect in Spain where the dialect is different.

Gorillas have also been taught how to communicate on various levels by humans. One very successful story is that of KoKo, a female gorilla. Scientists taught to her how to use sign language, which was considered to be quite a breakthrough with these animals. Gorillas have very good hearing so they can call out to each other. Koko can also communicate vocally. Perlman and Clark[6] after studying many videos of Koko, found that she voluntarily made nine distinct what are called vocal and breathing related behaviors, or VBB's.

The young definitely learn the voice of their mother at a very young age. They can pick up low noises that humans cannot hear easily and that is often how they are alerted to dangers. Young gorillas have communications that they use which are similar to those of human babies. They include whining and making sharp noises. Their mothers are quick to try to find out what they need to get those forms of communication replaced with those that are more along the lines of contentment.

Baboon

Baboons have a complex system of communication that includes vocalizations, facial expressions, posturing, and gesturing. These vocalizations, which baboons use to express emotions, include grunts, lip-smacking, screams, and alarm calls. The intensity of the emotion is conveyed by repetition of the sounds in association with other forms of communication.

Baboons communicate with each other primarily through body gestures and facial expressions. The most noticeable facial expression is an open-mouth threat where the baboon bares the canine teeth. Preceding this may be an eyelid signal, raising the eyebrows and showing the whites of the eyes, that is used to show displeasure. If a baboon really becomes aggressive, the hair may also stand on end, threatening sounds will be made, and the ground will be slapped. In response to aggressive facial expressions and body gestures, other baboons usually exhibit submissive gestures. A fear-face, a response to aggression, involves pulling the mouth back in what looks like a wide grin.

More vocal, though, Baboons make different sounds in order to communicate as well. One of the most common vocalizations given by olive baboons of all ages is the "basic grunt", heard throughout the day as the group spreads

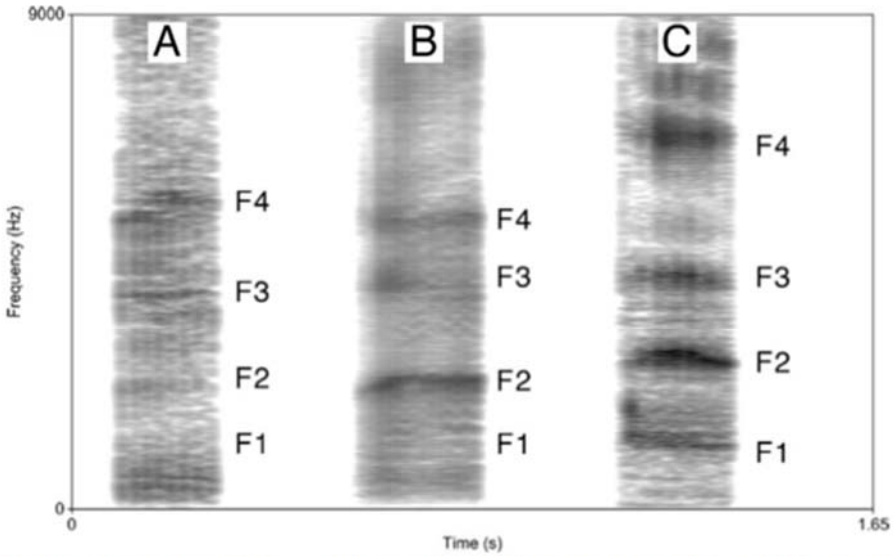


Fig. 4 Nasal grunts emitted by lemurs: *Eulemur fulvus* (a), *Eulemur macaco* (b), and *Eulemur rubriventer* (c). All spectrograms were generated in Praat (University of Amsterdam) using the following settings: view range, 0–9000 Hz; window length, 0.05 s; dynamic range, 50 dB.

out to feed, while traveling, during amicable social interactions, and as they settle into the sleeping site in the evening. Other vocalizations are heard under more specific conditions. Some calls given by adults include the "roargrunt", "cough-bark", and "cough geck". The "roargrunt" is heard in displays by adult males; this call is given through closed lips and is deep in pitch and sounds like a low humming. Given in situations of low anxiety, alarm, or discomfort, the "cough-bark" and "cough-geck" may be heard in response to unknown humans or low-flying birds. Other calls given by all group members include "two-phased barks" or "wa-hoos", "broken grunting", "pant-grunts", "shrill barks", and "screams". The "wa-hoo" is a two-syllable call given in series, ranging from one to 20 minutes in length in response to predators, during times of distress, such as when a baboon is separated from the group and trying to regain contact, and at night at the sleeping site in response to neighboring groups' "wa-hoos". Rendell *et al*.[7] found in their research that mothers could distinguish between individual baby calls for them, but not necessarily among stress screams if their young were being attacked by another group member.

Ring Tailed Lemur

Ring-tailed lemurs have 28 distinct call types, 22 of which are used by adults, six of which are particular to infants. Some predominant vocalizations include affiliative vocalizations such as "moans," contact calls used in conditions of low or moderate arousal, "meows," heard as contact calls in situations of moderate arousal or excitement and which are thought to increase group cohesion, and "wails" which are the highest arousal contact calls and are heard when a group member is separated from the social group. "Howls" are given only by non-infant males and are used to contact and advertise presence of the group to other groups in the area, "purrs" are heard during grooming and thought to be a sign of contentment, and "chirps" are given to elicit group movement from one location to another. Agonistic vocalizations include "yips," given by subordinate animals when approaching or when approached by a dominant individual, "squeals" are given

by males during displays to assert status over other males or when soliciting females, and "chutters," given when a dominant individual lunges at a subordinate individual. Ring-tailed lemurs also have specialized antipredator vocalizations that elicit responses from the rest of the group when they are given. For example, "gulps" are heard when a carnivore, raptor, or rapidly moving human are perceived and are generalized group alert vocalizations, "shrieks" are heard in response to large, low-flying birds, "clicks" are heard in situations of curiosity but wariness, and "yaps" are heard during mobbing of mammalian predators.

Figure 4 from Gamba *et al.*[8] shows that three different species of lemurs can be delineated by three different species-specific formant patterns.

Not So Large Terrestrial Mammals

Biggens[9] described the opossum's appearance, range, habitat, feeding habits, activity, reproduction, predators, and social behavior. Opossums hiss, screech, growl, and make clicking sounds to scare off predators. Their predators tend to be larger and faster than the possum and include bobcats, coyotes, and great horned owls. Listening to the recordings in the MLS library and then researching the behavior of the animals is helping us to connect the two. Fadem[10] conducted a study of opossums in captivity. She noted that the two main vocalizations, screeching and clicking, occurred under different circumstances. Screeching was indicative of a highly threatening situation whereas clicking was often used to subdue others or used when submissively retreating from another.

This does align with our original findings in our sound analysis. Our theory is that the sound being made by the possum was to defend itself, as it was being held captive. The groaning sounds sounded like clicking. This is characteristic of this creature trying to fend off predators. The growls, hisses, and screeches mentioned in that article do align with this aggressive behavior, exhibited in situations of danger.

The chimpanzee MLS audio recording had howling, screeching, and an overall aggressive tone. The chimpanzee vocalization did not follow a specific pattern, unlike that article which would suggest otherwise. However, this behavior, with relation to the article, may indicate that the chimpanzee was trying to get attention, since it was very vocal. The article cannot help us further, as it does not delve deeper into this behavior. However, the fact that there were two adults should indicate that they were communicating with one another; whether it was aggressive or friendly is unclear.

One theory is that the sound being made by the possum was to defend itself, as it was being held captive. The groaning sounds sounded like clicking. This is characteristic of this creature trying to fend off predators. The growls, hisses, and screeches mentioned in the article do align with this aggressive behavior, exhibited in situations of danger.

The rock hyrax, *Procavia capensis*, (a gerbil-like creature) is found most commonly in Africa or the Middle East in rocky terrains acres Israel. The current article[12] provides evidence of complex syntactic vocalizations in a small social mammal, the rock hyrax. Methods of syntactic analysis have rarely been applied to mammals and are more commonly used for bird song. Male rock hyraxes produce long, complex songs, lasting several minutes. The article presents four spectrographic

representations of four types of hyrax syllables which are: a. wail, b. chuck, c. snort, d. squeak.

Each hyrax song typically consists of of a sequence of syllables, followed by a short pause. The purpose of the male song is unclear but is thought to be a form of advertisement which is pretty interesting. I would have thought it would be used as a mating call/ song. Researchers found that because hyrax social structure is so complex that it usually the higher-ranked males that do most of the singing and other males appear to play a significant role in social activity of the group.

Some hyrax songs can be represented by a string of discrete syllables and this requires researchers to use techniques in other fields for the processing of digital information. In the article they used techniques specifically like bioinformatics which uses algorithms for the analysis of DNA sequences. Information theory for digital signal processing was also used to generate a number of metrics for measuring the information.

The researchers sampled hyrax songs in nine regions around Israel and each region contained two to nine sites. The sites were ecologically similar and near each other so hyrax migration could be possible. The interesting part of this research is that because higher-ranked males are those who carry out a majority of the singing activity, songs recorded at each site came from one or two hyraxes. So essentially they focused on the animal who had the strongest sound. In some ways this is funny because the animal makes the sound for attraction of other hyraxes and here it is attracting researchers. The researchers then divided the songs into syllables by visual inspection of the spectrogram and bouts were defined as a sequence of syllables bounded by a period of silence.

The data collected from the rock hyraxes song shows that a hyrax begins a song with very short bouts then they add more complexity as the song progresses. Out of the 3126 total bouts 19% or 559 bouts contained six or more syllables. Their survey size was about 1 to 55 bouts per site and the mean bouts was 15. Permutation tests show that sites within the same region were significantly more similar to each other than sites between regions.

What the researchers discovered is that the significance measured for different regions show that the rock hyrax does have distinct syntactic dialects among distant regions across Israel. This is amazing because the researchers also found that hyraxes in different regions of the country sing different songs that are very different from the syntactic repertoire in other[11].

Ten interesting things about the hyrax:

-The social classes of the rock hyrax
-Upper-class male rock hyraxes produce more song than any other rock hyrax
-The song of the rock hyrax varies depending on location
-It is a mystery why the hyrax makes its song; most likely thought to be for attraction
-Researchers recorded and analyzed the hyrax who sang the loudest and the most
-Hyrax songs can have syllables that cannot be heard by the human ear and need other technologies in order to pick them up.
-The five different types of rock hyrax syllables like the wail, chuck, snort, and squeak

-The fact that those categories would fall under song. Usually the term song is associated with birds or music not mammal vocalizations.

-Syntactic dialects in hyrax populations can evolve and be maintained by social learning, copying and alteration by improvisation of the order of song elements

-In rock hyrax, society, top-ranking males, which are often immigrants from nearby sites, sing more frequently

Birds

Bald eagles have weak-sounding calls which are usually a series of high-pitched whistling or piping notes. The female may repeat a single, soft, high-pitched note that signals her readiness for copulation---ki-ki-ki-ki-ki-ki-ker

After hatching, the nestlings make a single- note tonal peep[12]. As the bird ages, its sounds become more complex and have a greater volume variance. The cheeping call of the nestling serves as a way to beg for food, an alarm call, and a communication with adults (https://pages.vassar.edu/sensoryecology/bald-eagle-haliaeetus-leucocephalus-singing-behavior/).

Calls of Magellanic penguins include ecstatic display calls, mutual display calls, fight calls and contact calls. Females respond more strongly to their mates' calls than to other male calls. Males perform ecstatic display calls in the beginning of breeding season to attract a mate and during altercations with other males. Penguins vocalize to recognize each other. Each penguin's voice is as distinct as a human fingerprint.

Little penguins/Fairy penguins are highly vocal during the night while roosting. The sound of their calls can range from a low rumble to a trumpet-like noise. Their song can be used for several functions, including attracting mates. Each little penguin has a distinctive individual song that is used by parents and siblings to distinguish one another from strangers. Calls can also be used with an aggressive intent against an intruder around a penguin's nest. Calls are distinctive for each adult or chick and are used in bonding, courtship, defense of territory, aggressive behavior, and as a way to recognize each other. Grunts, roars, brays, and various beeps are used when the bird is in an aggressive mood. Chicks have a high-pitched beep that develops into adult vocalization close to time of fledging (http://www.aquariumofpacific.org/exhibits/penguin_habitat/magellanic_penguin).

In a study done with king penguins, it was found that males incubating eggs could effectively discriminate returning mates with acoustic calls (70% of the time on the first call)[13].

Great Gray Owl

According to Brunton and Pittaway[14], the owls emitted sounds where "...one was a short, rasping *e-e-e-e-e-e-e-e-e-e* which did not carry far. The other was a quiet, drawn out *Who-oo-oo-oo-oo-oo*." Another time, when the owl was a short distance of 250 feet away from another bird, it gave a "...short, rasping call, followed by a drawn-out *Who-oo-oo-oo-oo*. The rasp was given once and then the *Whoo-oo-oo* given three times". These vocalizations were mainly used to establish territory, and home range, and that the owl was purposefully directing these calls at the other bird. The time intervals in which the owl made the sounds/calls were recorded, as well as the distance between the owl and the

other bird. Other observations gleaned from reading the article were as follows:

- Great Gray Owls do not seem to be particularly vocal in the Canadian Winter Range.
- Many of the vocalizations made by the Great Gray Owl are very similar to other birds such as the Great Horned Owl, the Sparrow Hawk *(Falco sparverius)*, and the Northern Shrike *(Lanius excubitor).*
- In this particular study, though the owl makes attempts to exert control over its territory; it does not seem to act on it, but rather sits there passively while making the calls.
- It actually did nothing to show it considered the other bird an enemy.
- There seemed to be a particular pattern to the calls it made to the other bird, making the “Who-oo-oo” sound three times after rasping once.
- The Great Gray Owls on their own, seem to show great interest and curiosity in the other birds of their species, especially when they’re hunting.
- They are particularly vocal when it comes to territorial clashes with other Great Gray Owls.
- However, they seem to be very passive when dealing with other birds of other species regarding their territory, they only rasp and hoot a couple of times; they do not screech or raise their volume.
- When they do confront other birds about their territory, they angle their sounds directly towards the other bird.
- Because these calls and rasps are directed towards one particular birds, other birds in the area who are doing the same thing will not respond, and will continue what they were doing.

White Throated Bee Eater

Not much scholarly information can be found on White-Throated Bee-Eater sounds. Some vocalizations from the ML recordings can be described as "rolling and liquid quality". In general for all bee-eaters, there are common contact calls, plus greetings, appeasement vocalizations, threat calls, predator alarms (“waark”), vocalizations during courtship feeding and feeding of young. The most common call is a muffled nasal “gaaa” and a shrill “kwannk” sound. They also make a slightly rolled "krrt" or "karara."

Amphibians

Amphibians are also animals that do not immediately come to mind when recording vocalization---partially because many are nocturnal. For example, if one tried to record vocalization of frogs at the Houston Zoo, one might be disappointed (Nolan, personal communication).

The purple frogs of India make vocalizations that are interesting, short and frequent (vibration) calls because they are earless frogs that live and breed in the soil[14]. These purple Indian frogs make two to six short pulsatile calls ranging from five to seven seconds each. The vocalizations they make are very similar to those of other frogs, despite that these frogs are a rare species. The calls were measured at 106 pulses/s and the call’s dominant peak was between 1200-1300 Hz with no frequency modulation.

The scientists used this information to learn more about the male frogs’ interactions with their neighbors, as well as to learn more about what happens to the temperature, body size, and overall condition of the frogs when they make the calls. Frogs use the calls for mating. Figure 5 from Thomas[15] shows

that the vocalizations are much shorter in duration than sea lion barks and that this can be visualized through the sound waves recorded and subsequent spectrograms.

Figure 5 (Figure 2 in Thomas[15])

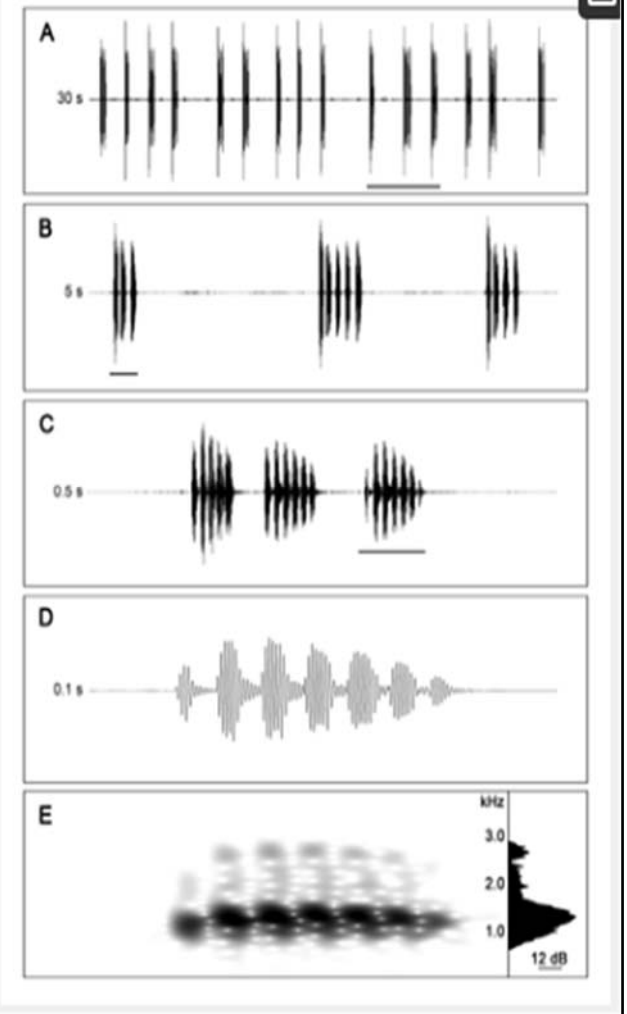
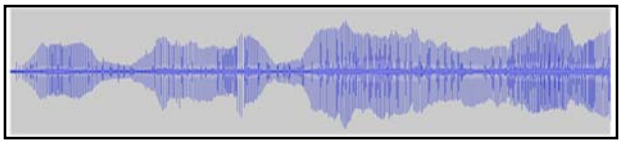


Figure 2. Advertisement calls of a male *Nasikabatrachus sahyadrensis*.

(a) 30-s segment of continuous, spontaneous calling by a single male. (b) 5-s segment showing the three consecutive call groups underlined in (a). (c) 0.5-s segment showing the three calls of the call group underlined in (b). (d) 0.1 s segment showing the call underlined in (c). (e) Spectrogram of the call illustrated in (d); *Inset*: power spectrum averaged over the duration of the call depicted in (d). The entire 30-s segment depicted in this figure is included as an audio file in the Supporting Information for this article (Audio S1).http://dx.doi.org/10.1371/journal.pone.0084809.g002

Future research

Nestor[16] very recently posted a recording of a sperm whale. We were able to record the sounds, mainly a series of clicks, in Audacity, as shown below. The clicks are thought to be sonar waves that can be used to detect food. Since walruses and stellar seals (and sometimes sea lions) also make clicks, it would be interesting to learn more about his.



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Streptococcus mutans Biofilm Inhibition by Miswak and Green Tea

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Abstract

Bacteria growing in biofilms demonstrate higher resistance to antimicrobial agents and the immune response than their planktonic counterparts. *Streptococcus mutans* is among the earliest bacterial colonizers existing on the tooth surface biofilm, known as dental plaque. Within this total bacterial community, *S. mutans* functions by lowering the oral pH. This leads to erosion of enamel and causes dental caries. Previous studies in various ethnic cultures have demonstrated the antibacterial nature of natural plant-based remedies including miswak *(Salvadora persica)* and green tea *(Camellia sinensis*). Research students at Saint Peter's University hypothesized that there would be a similar inhibition on *S. mutans* biofilm formation and evaluated the effect of these agents. *S. mutans* biofilms were grown under microaerophilic conditions, in a sucrose based-minimal media, using 24-well polystyrene plates. During development, biofilms were exposed to various concentrations of miswak (0-2% w/v) and brewed green tea (0-10% v/v). Following incubation, wells were quantified for biofilm formation, using a standard crystal violet assay. Each experimental condition was incubated in triplicate and the experiment was repeated twice. Biofilms treated with miswak exhibited a (-0.99-fold, P= 0.0017) change in biofilm mass when added at concentrations ≥2% w/v, and inhibited in a dose-dependent manor when added between 1% w/v (-0.86-fold, P=0.006), and 0.5% (-0.6- fold, P=0.006). Addition of brewed green tea to the developing biofilm also exhibited a dose dependent inhibition with a (-0.95-fold, P<0.0001) change at values ≥10% v/v, (-0.78-fold, P<0.0001) change at 5%, (-0.3-fold, P=0.0052) change at 2.5 %, and a (-0.17-fold, P=0.024) change at 1.25%, relative to the untreated control. Our findings support the inhibition of cariogenic *S. mutans* biofilms by miswak and green tea. These natural plant-based remedies may provide necessary caries biofilm infection control in many areas of the world.

Introduction

Biofilms and Oral Streptococci

Biofilms are organized communities of surface-adherent microorganisms embedded in an exopolysaccharide matrix (EPS). The components of the EPS contain a wide variety of polysaccharides, proteins, glycoproteins, and extracellular DNA. Biofilms consist of dense clusters of bacterial cells which aggregate together. The formation of biofilms occurs in a series of distinct steps including initial adherence, irreversible attachment, maturation, and dispersion[1,2]. Unlike planktonic (free-living) cells, bacterial cells living within a biofilm may exhibit increased resistance to antimicrobial agents and the immune response of the host[3]. Biofilms may consist of one species of bacteria or several species, as in the case of dental plaque[4]. Within the mouth, the oral plaque

biofilm comprises more than 700 species, of which approximately 20% are streptococci[5]. Streptococci are among the earliest colonizers on a freshly cleaned tooth surface[6,7]. Adherence occurs through specific receptor-ligand interactions on the bacterial cell surface and the acquired pellicle consisting of salivary glycoproteins[8] including salivary agglutinin and sialylated mucins[6]. Important streptococcal early colonizers include *S. oralis*, *S. sanguinis*, *S. gordonii* and *S. mutans*. *S.mutans* is the leading cause of dental caries[9]. In this infection, *S. mutans* forms sticky oral biofilms that adhere to a salivary glycoprotein-coated surface[10], leading to the production of lactic acid which erodes the enamel and causes progressive tooth decay. Dental caries is a significant contributor to health care costs in both industrialized and developing nations. Dental caries is the second most common disorder world-wide; affecting approximately ninety-two percent of adults aged twenty to sixty-four. In the United States, dental caries incidence is five times more prevalent than asthma and is the most common reason for tooth loss among children[11].

Within the plaque biofilm, the majority of oral streptococci, including *S. mutans,* exhibit genetic competence, which is the ability to acquire and incorporate free exogenous DNA in their genome[4]. Genetic competence is believed to be instrumental for bacteria to adapt to their environment and often contributes to an increase in clinical severity, as in the case of uptake of antibiotic resistance genes. Examination of the *S. mutans* competence genes has shown levels of increased expression when grown in a biofilm relative to planktonic conditions. Additionally, this analysis supported uptake of resistance genes from nearby dead bacterial donors[12,13].

Prevention of biofilm formation in the cariogenic species *S. mutans* in the oral cavity is necessary for proper oral hygiene. In many regions of the world chlorhexidine rinses are used as the “gold standard” in oral anti-cariogenic treatment. Chlorhexidine functions by destabilizing the peptidoglycan within the bacterial cell wall and interfering with bacterial osmosis. The uptake of chlorohexidine by bacteria is very rapid, and upon entry chlorohexidine ruptures the cell wall and cytoplasmic membrane leading to cell death[14]. However, many side-effects have linked to chlorohexidine usage, including discoloration of teeth, altered taste sensation, mucosal irritation, parotid gland swelling, and enhanced supra-gingival calculus formation, therefore limiting its effective use as a therapeutic agent[15]. Due to the evidence of abundant genetic exchange observed in plaque biofilm, the rapid evolution of antibiotic resistance among microorganisms[16], and side effects of many commercially available mouth rinses, it has become increasingly important to find alternative and natural treatments for their control. In many regions of the world, natural agents have long been used to combat bacterial infections, including medicinal plants which have been used for much of recorded history and within specific cultures. Many of these agents and their synthetic derivatives have been shown to possess chemical substances capable of fighting certain bacterial species. This study aimed to examine the inhibitory effect of natural, plant-derived, remedies on biofilm formation in *Streptococcus mutans* using a standard crystal violet assay[17,18]. The agents tested were selected by research students at Saint Peter’s University, based upon cultural and familial preferences, and included miswak (*Salvadora persica)* chewing sticks and green tea *(Camellia sinensis*).

Miswak

Throughout history, humans have incorporated a variety of mechanisms for oral care, including both mechanical and chemical means. The recorded account of human oral hygiene dates may be traced far back as 3500 B.C.E. by the Babylonians[19]. During this time tooth cleaning instruments included twigs and tree fiber brush. The tree fiber brush, now known as the chewing stick, was the prototype for modern toothbrush[20]. Chewing sticks are still extensively used throughout many parts of Africa, Asia, South America, and the Middle East. Of the approximately one hundred and eighty plant species harvested for use as a chewing stick, miswak (*Salvadora persica)* is the most commonly used, due its relative softness[20]. During harvest the fresh young stems of the shrub are collected. The word "miswak" is Arabic for chewing stick. In many locations throughout the world, its use is a cultural tradition rather than a clinical preference. The use of Miswak is often compulsory within the Muslim world and dates prior to the prophet Mohammed, who praised its usage[21]. Numerous clinical investigations of Miswak have supported its anti-cariogenic effects, with some studies demonstrating levels of caries prevention even greater than the traditional toothbrush[22-24]. Due to its effectiveness, easy accessibility, and low cost, the World Health Organization has approved the chewing stick as an appropriate means of oral hygiene[25].

Chemical analysis of *Salvadora persica* has revealed many beneficial active phytochemical agents which contribute to its antibacterial properties. These include sulfur, alkaloids, fluoride, tannins, and benzyl isothiocyanate[26]. The novel alkaloid present within *S. persica* has been identified as Salvadorine and cleavage yields trimethylamine, which exhibits a bactericidal effect. In addition, *S. persica* extract contain Vitamin C, which aids in tissue healing and repair, and silica, which is known to prevent staining of teeth[26].

Green Tea

After water, tea is the second most commonly consumed beverage worldwide[27]. Green, black and oolong tea are derived from the *Camellia sinensis* plant and differ only in the level of fermentation during processing. Black tea is fermented, oolong undergoes partial fermentation, and green tea is unfermented[28]. Green tea's uses are very versatile and can be brewed, taken in capsules, and used in skin products. Green tea is a popular beverage worldwide, particularly in Asian countries, and boasts many health benefits. Its medicinal use in China dates back at least 4000 years with an ancient Chinese proverb that states 'Better to be deprived of food for three days, than tea for one'[28]. Although the chemical composition of green tea may vary with climate, season, and agricultural practices, the active beneficial ingredients include the polyphenols. They are the catechins: epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). ECGC is believed to be the most significant of these components. This potent antioxidant is known to inhibit the growth of cancer cells, without harming healthy cells. Numerous studies have also demonstrated the efficacy of green tea in lowering LDL cholesterol and inhibiting abnormal clot formation, thereby preventing heart attacks and strokes[29-30]. Consumption of green tea has also been

linked to antimicrobial effects and has been shown to strengthen the immune system. Green tea-based oral rinses inhibit levels of cavities and gingivitis in both adult and children[30,31]. It is postulated that the bioactive components of green tea may interfere with bacterial proliferation and adhesion to tooth enamel[32,33]. Other compounds of interest in dried green tea leaves include quercetin (known for its anti-inflammatory effects), kaempferol (anti-carcinogenic), myricetin (known to lower cholesterol and be anti-carcinogenic), and chlorogenic acid (antibacterial, antioxidant, and anti-carcinogenic effects)[34,35]. Because miswak and green tea have shown ease of accessibility and benefits for caries prevention, we sought to examine their effect *in vitro* on *S. mutans* early surface adherence and biofilm formation, using polystyrene wells to represent the freshly cleaned tooth surface.

Methods

Microbial culture

S. mutans (ATCC 25175) was grown at 37°C under reduced oxygen conditions using a candle jar. Planktonic growth occurred within Brain Heart Infusion (BHI) broth. Biofilm growth induction occurred in a minimal biofilm media (BM) (A kind gift from Dr. Todd Kitten, Virginia Commonwealth University, manufactured by SAFC Biosciences, Lenexa, KS) supplemented with 1% w/v sucrose (BMS). These conditions were previously demonstrated to induce robust biofilm formation in the oral streptococci[36].

Preparation of Anti-biofilm treatments:

Miswa: Two grams of hygienically-sealed miswak (Al-Rashad LLC, Product of Pakistan), were crushed within a sterile Falcon tube using ethanol flame-sterilized scissors and added to freshly prepared BMS, to create a final volume of 10 ml. The sealed mixture was then vortexed until the miswak dissolved within the BMS media, to create a 20% w/v aqueous solution. To test for the Minimal Inhibitory Concentration (M.I.C.), two-fold serial dilutions of the 20% mixture were then created resulting in 10% w/v, 5% w/v, and 2.5% w/v.

Green Tea: To examine levels of green tea similar to those normally consumed in beverage form, a 1.9 g (bag) of organic green tea (East West Tea Company, Springfield, Oregon) was steeped in 250 ml sterile 100°C water for 5 minutes. The tea was then cooled, and, to ensure sterility, was filtered using a 0.22 μm filter. To also determine an M.I.C., two-fold serial dilutions were prepared in sterile BMS resulting in 50% v/v, 25% v/v, and 12.5% v/v concentrations relative to the brewed tea. Both treatment agents were assessed relative to an untreated negative control, BMS, and a positive antibiotic control, a 30 μg tetracycline disc (TE-30 Fisher Scientific). Sterility of both test agents was assessed by overnight growth with no added bacteria.

Crystal Violet Biofilm Assay Using Polystyrene Microtiter Plates

To examine the effects of the miswak and green tea treatments on *S. mutans* biofilm development *in vitro,* the previously described crystal violet biofilm assay[17,18] was utilized. Using aseptic technique, 900 μl of freshly prepared BMS was added to each well in a sterile 24-well polystyrene plate (Thermo Scientific™ BioLite Microwell). As illustrated in Table 1 (adapted from

Table 1. 24-well plate configuration for Miswak treatment					
A	**B**	**C**	**D**	**E**	**F**
Tetracycline *S. mutans*	BMS *S. mutans*	2% miswak *S. mutans*	1% miswak *S. mutans*	0.5% miswak *S. mutans*	0.25% miswak *S. mutans*
Tetracycline *S. mutans*	BMS *S. mutans*	2% miswak *S. mutans*	1% miswak *S. mutans*	0.5 % miswak *S. mutans*	0.25% miswak *S. mutans*
Tetracycline *S. mutans*	BMS *S. mutans*	2% miswak *S. mutans*	1 % miswak *S. mutans*	0.5 % miswak *S. mutans*	0.25% miswak *S. mutans*
Tetracycline *Uninoculated*	BMS *Uninoculated*	2% miswak *Uninoculated*	1 % miswak *Uninoculated*	0.5 % miswak *Uninoculated*	0.25% miswak *Uninoculated*

Table 2. 24-well plate configuration for Green Tea solution treatment (Seen in Fig.1)					
A	**B**	**C**	**D**	**E**	**F**
Tetracycline *S. mutans*	BMS *S. mutans*	10% green tea *S. mutans*	5% green tea *S. mutans*	2.5% green tea *S. mutans*	1.25% green tea *S. mutans*
Tetracycline *S. mutans*	BMS *S. mutans*	10% green tea *S. mutans*	5% green tea *S. mutans*	2.5 % green tea *S. mutans*	1.25% green tea *S. mutans*
Tetracycline *S. mutans*	BMS *S. mutans*	10% green tea *S. mutans*	5 % green tea *S. mutans*	2.5 % green tea *S. mutans*	1.25% green tea *S. mutans*
Tetracycline *Uninoculated*	BMS *Uninoculated*	10% green tea *Uninoculated*	5 % green tea *Uninoculated*	2.5 % green tea *Uninoculated*	1.25% green tea *Uninoculated*

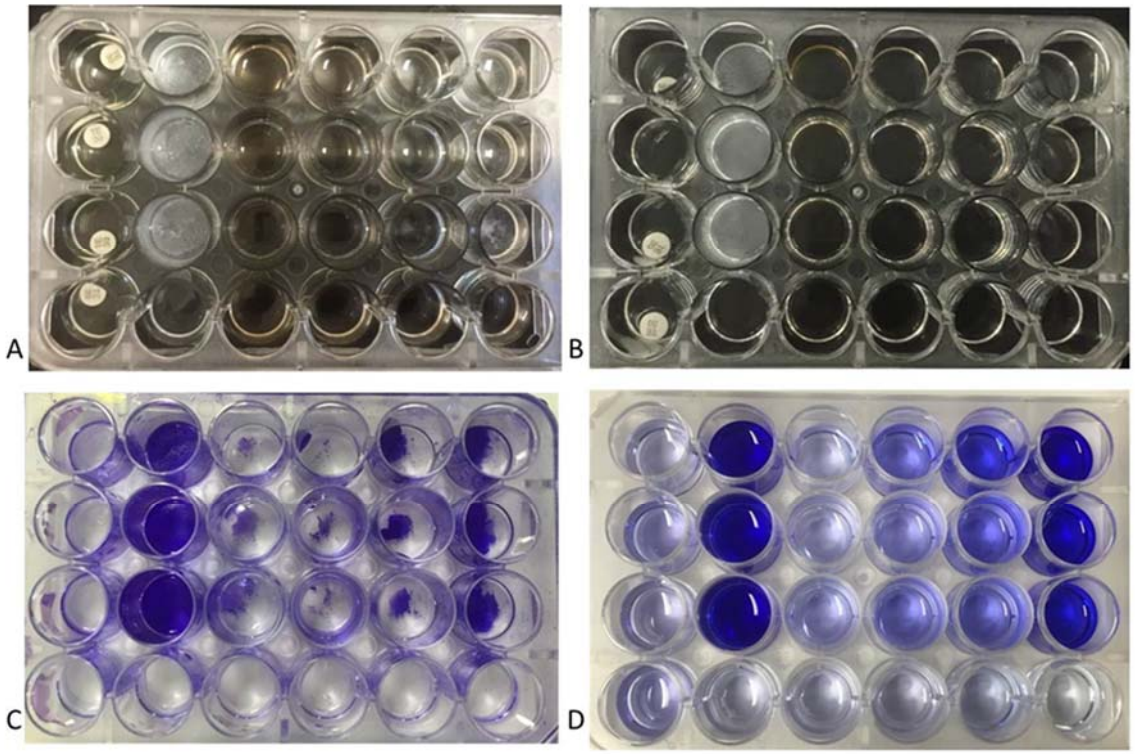

Figure 1. Polystyrene Microtiter Assessment of a Representative 20-hour *St. mutans* biofilm plate treated with Green Tea (Contents Indicated in Table 2)

a) Biofilms prior to decanting planktonic cells.
b) Biofilms following decanting planktonic cells.
c) Dried biofilms stained with Crystal Violet
d) 30% glacial acetic acid solubilized, stained biofilms.

Callahan and Castaldi, 2016)[37], 100 µl of BMS plus a TE-30 disk was added in quadruplicate to the wells in Column A, to serve as a biofilm inhibiting positive control. In Column B, 100 µl of BMS was added in quadruplicate to the wells to serve as a negative control. 100 µl of each treatment agent was added in quadruplicate to each well in respective columns C-F. This additional 10-fold dilution resulted in miswak concentrations of 2%, 1%, 0.5% and 0.25% (all w/v) in the plate. Lastly, 10µl of overnight culture was added to each well in the top 3 rows. The bottom row was not inoculated to test for contamination. The same procedure was carried out for the green tea solution biofilm plate as indicated in Table 2 with 10%, 5%, 2.5%, and 1.25% (all v/v). Each experiment was repeated twice.

Biofilm Viewing and Staining

The plates were then covered and incubated in a candle jar at 37°C for ~ 20 hours. The following day the 24-well plates were removed from the incubator. Figure 1a illustrates a representative green tea plate described in Table 2, following incubation. The planktonic growth was then carefully decanted and excess liquid was blotted with paper towel. Each well was rinsed twice with 1 ml sterile distilled water. The excess water was again removed by blotting dry into paper towel. The plate was then dried with lid off for approximately 15 minutes in 37º C incubator (Figure 1b). To stain the biofilms, 300 µl of a 1% crystal violet (CV) solution (prepared in 100% ethanol) was added to each well of the plate, and incubated at room temperature for 15 minutes. The CV was then carefully decanted into a waste tray. The plate was again rinsed three times by adding 1 ml sterile distilled water to each well. The plate was then uncovered and placed upright in the 37º C incubator to dry for approximately 20 minutes (Figure 1c).

Biofilm Solubilization and Absorbance readings

To quantify biofilm levels, the dried CV in each well was solubilized by adding 1.5 ml 30% glacial acetic acid (v/v) (Fig. 1d). 1 ml from each well of the solubilized CV was then added to the same location in a duplicate plate. To create the blank for each condition, 1 ml acetic acid solubilized CV from each un-inoculated condition was tested against its respective un-inoculated wells. 1 ml from each treated well was added to a cuvette and absorbance of solubilized biofilms was then read at 600 nm using a GENESYS 20 spectrophotometer.

Statistical Analysis and Assessment

Mean absorbance reading and standard deviations from three independent experiments were calculated using Graph Pad Prism. Mean values were proportional to amount of biofilm in each well. An unpaired, two-tailed T-test was performed to determine which treatments significantly reduced biofilm formation, relative to an untreated control.

Results

As seen in Figure 2 and Table 3, a ≥ 2% w/v aqueous solution of miswak incubated with *S. mutans* significantly inhibited -0.99-fold, (p= 0.0017) biofilm development at levels similar to samples that were with tetracycline. Inhibition occurred in a dose-dependent manor when miswak was added between 1% w/v -0.86-fold, (*P*=0.006), and 0.5% w/v -0.6-fold, (*P*=0.006). The MIC value appeared

to occur at a range between 0.5% w/v and 0.25% w/v. At levels ≤ 0.25% there was no significant inhibition relative to the untreated control. In a separate study, addition of 10% v/v Listerine™ to developing *S. mutans* biofilms also inhibited at levels similar to TE-30 (data not shown).

Figure 2. Crystal Violet Absorbance Assay of *S. mutans* biofilms treated with Miswak

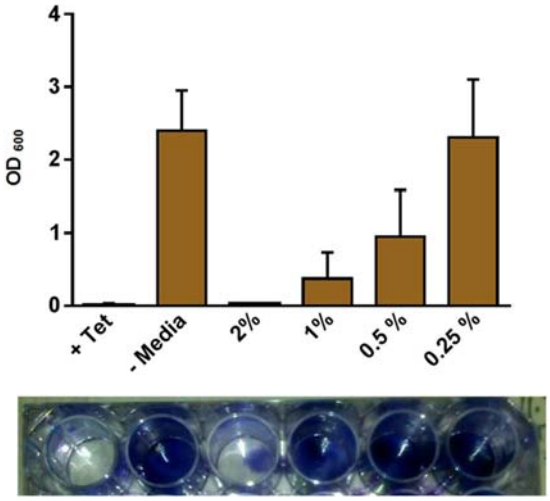

Figure. 2. Absorbance means of solubilized CV biofilms + SD from three independent assays, in which developing *Streptococcus mutans* biofilms were incubated with various amount of miswak in triplicate wells. Values indicated are aligned with a representative stained well of each condition on a dried plate prior to solubilization. Mean values are proportional to amount of biofilm in each well.

As seen in Figure 3 and Table 4, incubation of a developing *S. mutans* biofilm with an aqueous solution of brewed green tea inhibited *S. mutans* biofilm formation in a dose-dependent manner. When the green tea treatment was introduced at levels ≥10% v/v there was a -0.95-fold, (P<0.0001) change in biofilm formation, a (-0.78-fold, P<0.0001) change was observed at 5% v/v, -0.3-fold, (P=0.0052) change at 2.5 %, and a -0.17-fold, (P=0.024) change at 1.25%, relative to the untreated control. The MIC appears to occur at a level < 1.25%. No significant difference was seen in biofilm inhibition between incubation with TE-30 and ≥10% v/v brewed green tea (p= 0.3424).

Figure 3. Crystal Violet Absorbance Assay of *S. mutans* biofilms treated with Brewed Green Tea

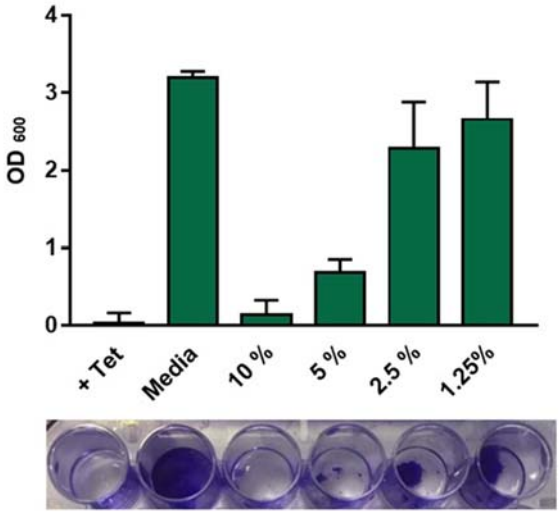

Figure. 3. Absorbance means of solubilized CV biofilms + SD from three independent assays, in which developing *Streptococcus mutans* biofilms were incubated with various amount of green tea solution in triplicate wells. Values indicated are aligned with a representative stained well of each condition on a dried plate prior to solubilization. Mean values are proportional to amount of biofilm in each well.

Condition	+ Tet	- Media	2%	1%	0.5 %	0.25 %
Mean	0.019	2.399	0.026	0.375	0.947	2.305
SD	0.013	0.451	0.005	0.294	0.524	0.652

Table 3. Mean Absorbance readings ± SD of solubilized Crystal Violet of *Streptococcus mutans* Biofilms treated with TE-30 discs, no treatment, and indicated amounts of Miswak

Condition	+ Tet	- Media	10 %	5 %	2.5 %	1.25%
Mean	0.058	3.195	0.185	0.687	2.285	2.657
SD	0.048	0.084	0.131	0.168	0.595	0.486

Table 4. Mean Absorbance readings ± SD of solubilized Crystal Violet *Streptococcus mutans* Biofilms treated with TE-30 discs, no treatement, and indicated amounts of brewed, cooled, and filtered green tea.

Discussion

This study supports the efficacy of miswak and green tea in inhibiting *S. mutans* biofilm formation. Our findings are in agreement with previous clinical studies of dental patients using miswak and green tea treatments. It is currently estimated that 60-80% of bacterial infections are biofilm in nature[38]. With the increase in bacterial resistance, and evidence of genetic exchange in the oral cavity biofilm, it is becoming increasingly important to find remedies that will inhibit bacterial biofilm proliferation. In examining the efficacy of natural remedies, the crystal violet microtiter plate assay of O'Toole[17,18] is a very efficient tool for student-selected treatment agents within a laboratory setting and can be performed with a variety of bacterial species. Furthermore, this study has demonstrated, within an undergraduate research setting, that allowing student input in selecting natural agents to examine against bacterial biofilm formation has become a useful engagement tool in the students' motivation toward their research projects. Many students have examined time-honored family remedies and showed a vested interested in the study findings.

Plant-based treatments offer many advantages over harsh chemically-based agents and antibiotics, known to have many harmful side effects. During the course of this study, the authors tested miswak for personal oral hygiene and noted its bitterness, which may have resulted increased saliva flow. This effect may facilitate the release of necessary enzymes such as lysozyme thereby assisting in the inhibition of oral bacteria[39]. The miswak chewing stick is amongst the oldest form of teeth cleaning, and it has proven to be clinically effective, economical, simple to use, and easily accessible in areas where access to dental treatment may be limited. In addition, the ancient use of green tea for oral rinsing to aid in cavity prevention was supported at the level of biofilm formation. Future studies are planned to examine the effect of black tea and oolong tea on *S. mutans* biofilm formation.

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Are Rodlet Cells Reliable Biomarkers in *Fundulus heteroclitus* (L.)?

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Abstract

Two populations of killifish, *Fundulus heteroclitus* (L.) were studied, one from a high environmentally impacted site (Saw Mill Creek) exposed to pollutants, heavy metals and infected with parasites, the other from a habitat that was far less affected (Lemon Creek), on Staten Island, New York. The rodlet cell counts in the gills of each population were compared and correlated with the number of gill parasites. A comparison of rodlet cell numbers was also made between mature males and females within each population. Although the parasite infestation was significantly higher in the Saw Mill Creek population, the rodlet cell counts did not differ between the two groups. There was no statistical correlation between the parasite number and number of rodlet cells or between standard length and rodlet cells in each population. Also, the number of rodlet cells did not differ between males and females in either group. Furthermore, EM observations showed no difference in the activity of these cells. Our findings suggest that in *F. heteroclitus*, rodlet cells are not dependable biomarkers for evaluating the fish's response to parasites and environmental stressors. In addition, the sexual status of the fish does not appear to affect the number of rodlet cells.

Key words: *Fundulus heteroclitus*, rodlet cells, gills, parasite, biomarker

Introduction

The rodlet cell (RC) has posed a conundrum for fish biologists since its identification over a century ago by Thelohan[1]. Found within viscera and epithelium of both freshwater and marine species[2-6], two major hypotheses emerged as to the origin and function of these cells. According to the exogenous hypothesis, this cell is a parasite; most likely of the Apicomplexa order[4,7] whereas the tenet of the increasingly more prevalent view is that the RC is an endogenous element. Although several functions have been assigned to the RC over the years (see review by Manera and Dezfuli[8]), growing evidence suggests that this cell plays a role in the fish's innate immune response[9-17], presumably as a member of the granulocytic line.

Studies on the role of RCs as immune effector cells have focused primarily on their mobilization and recruitment in response to parasite invasion[9-12,18-20], bacterial infection[21,22], stressors[23,24,25], environmental toxicants[26-29] and pathological lesions[30,31] . Exposed tissues including gills[27,29], intestine[10], skin[23,24], liver[11], kidney[9,18] and brain[20] invariably

showed a greater number of RCs than uninfected or unexposed fish of the same species. Thus, measurements of the response of RCs to various perturbations have come to serve as biomarkers for the potential negative impact of the environment on the health of the fish.

The gills of fish are often described along with skin, as the structures most often affected histologically by waterborne biological and physical contaminants[32,33] because these elements have the most direct and continuous contact with the aquatic environment. The focus of this study was to evaluate the role of RCs as dependable biomarkers in two populations of the estuarine killifish, *Fundulus heteroclitus* on Staten Island, New York exposed to two different levels of contamination. The Saw Mill Creek population had been exposed to a major oil spill that took place in 1990[34]. Studies showed the presence of high levels of Cu, Hg, Pb and Zn as well as organic pollutants at this site[34-37]. In addition to heavy metal exposure, the gills of these fish were heavily laden with trematode and myxosporean parasites. The Lemon Creek group was collected from a low impacted site located along the south shore of the island. Only an occasional myxosporean parasite was observed in the gills of these fish; trematode cercaria were never seen. The RC numbers were compared between males and females within each group and between groups. Correlations were made between parasite counts and the number of RCs within each population. We found that the RC numbers did not differ between males and females in either population and correlations between parasites and RCs were insignificant. Our results suggest that RCs are not influenced by sex hormones in mature fish. Furthermore, while RC recruitment serves as a good biomarker in the gills of other fishes exposed to hostile environments[8,12,29], our results indicate that they are not reliable biomarkers in *F. heteroclitus*.

Materials and Methods

Fish

Adult *F. heteroclitus* were collected during July and August, 2009 and 2013 using a common minnow trap from two collection sites, Saw Mill Creek and Lemon Creek, on Staten Island, NY. Spawning in this species commences in spring (March to May) and ends in late summer (July to September)[38]. The Saw Mill Creek collection (n= 13; four males, StL 64.1 $\pm$ 2.02 mm and nine females, 70.2 $\pm$ 4.26 mm) was from a highly contaminated site historically exposed to smelting activities[39] and subjected to an earlier major oil spill[34]. Previous studies showed high levels of heavy metals Cu, Hg, Pb and Zn as well as organic pollutants within the sediment[35-37]. The Lemon Creek population (n= 20; eight males, 44.4 $\pm$ 2.7 mm, ten females 46.3 $\pm$ 4.1 mm and two undetermined, 37.8 $\pm$ 5.2 mm) was collected from an area that is one of the few undisturbed tidal marshes on the island[37].

In the laboratory, each fish was sacrificed by over anesthetization in a solution of MS-222 (tricaine methanesulphonate (Sigma)) and its standard length measured to the nearest 0.1 mm.

Tissue preparation

Under a dissection microscope, the opercular plates were carefully cut away and the individual gill hemibranchs were separated and placed into Bouin's fixative for 24 hours. The tissues were

subsequently decalcified in a commercial 'decal' solution. In order to confirm the sexual status of the fish, the gonads were carefully removed via an anterior-posterior incision along the ventral midline followed by two vertical cuts at the anterior and posterior margins of the incision. The flap of tissue was peeled back to expose the viscera including the gonads which were carefully extracted and placed into Bouin's. Tissues were processed for paraffin in a routine manner. The gills were embedded so longitudinal sections could be cut ensuring the presence of the cartilaginous base support of the primary lamellae. Our previous work has shown the perichondrial area immediately beneath the afferent branchial artery to be a robust site of rodlet cell recruitment in *F. heteroclitus*[40]. Serial sections, 6 μm thick were cut with a rotary microtome, mounted on gelatin coated slides and stained with Masson's trichrome.

A small piece of each hemibranch was cut and processed for TEM according to the procedures previously described[41]. Sectioning was performed on a Leica Ultracut UCT microtome. Thick (1 μm) sections were stained with toluidine blue. Thin sections (80-100 nm) were collected on uncoated grids and stained with saturated aqueous uranyl acetate followed by lead citrate. Observations were made at 80kV on an FEI Tecnai Spirit transmission electron microscope.

Cell counting

Rodlet cell counts were made at the light level from ten randomly selected areas (151,976 μm^2) in the hemibranchs of each fish according to the procedures of Manera, Simoni and Dezfuli[14], stained with Masson's to accentuate cell identification. The total number of parasites was counted in every tenth section of the hemibranch of each fish. This precluded the possibility of counting the same parasite more than once.

Statistics

The mean rodlet cell counts of males v. females from SMC were compared using Student's T ($p < 0.05$) while in the LC group a one-way ANOVA was performed. Comparisons between groups were made with ANOVA. The mean parasite counts within and between populations were similarly analyzed ($P < 0.05$). Correlations between StL and RCs as well as parasite number and RC counts in both populations utilized Pearson's Correlation followed by least squares regression analysis ($p<0.05$) performed with Microsoft Excel.

Results

The gills of fish from the heavily impacted SMC population invariably showed marked signs of histopathology including extensive hyperplasia of interlamellar epithelium leading to fusion of secondary lamellae, abundant mucous cells, epithelial detachment in the secondary lamellae causing intralemellar edema and an increase in melanocytes and melanin deposits. Myxosporean cysts and trematode metacercaria were abundant in this tissue (Figures 1-3). The males averaged 19.7 ± 5.9 and the females 17.6 ± 3.37 parasites/section (table I). In contrast, the gills of fish from the LC site were normal in appearance with well defined secondary lamellae and a modicum of melanocytes and mucus producing cells. Parasites were scarce (Figures 4 and 5). The males averaged 0.21 ± 0.12, females 0.18 ± 0.05 and unknowns, 0.27 ± 0.2 parasites/section

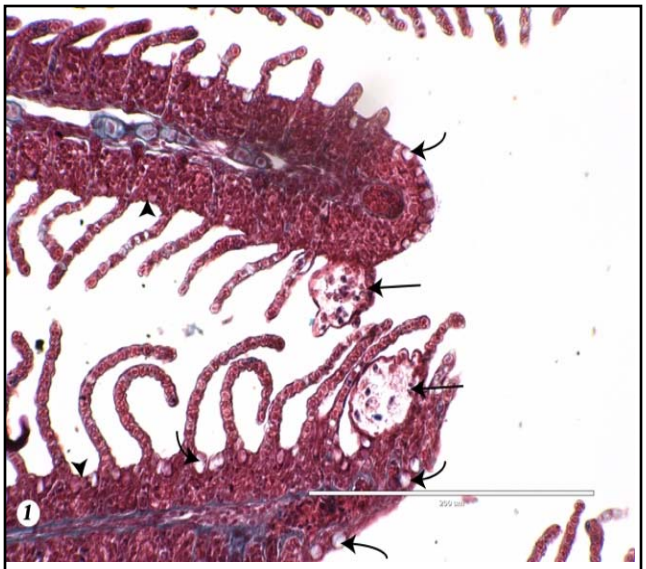

Figure 1. A section through the gill of an SMC fish. Note the presence of myxoporean parasites (arrows), hyperplasia of the interlamellar zone (arrowheads) and mucous cells (curved arrows).

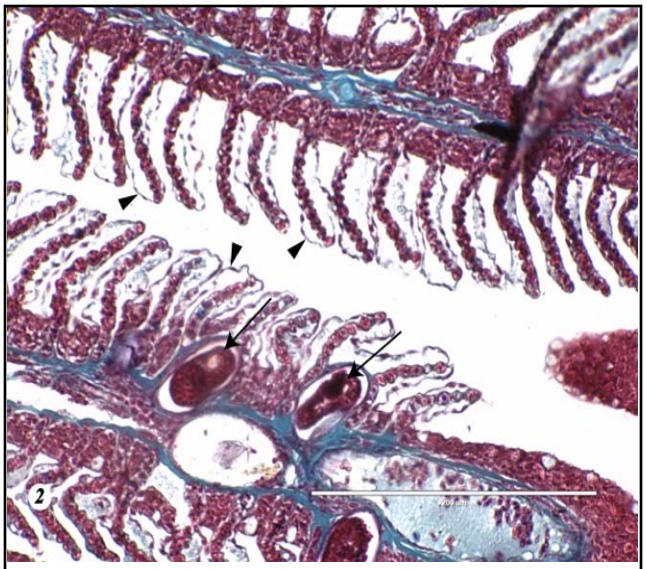

Figure 2. A section through the gill of an SMC fish infected with metacercaria cysts (arrows). Epithelial detachment of the secondary lamellae (arrowheads) is also apparent.

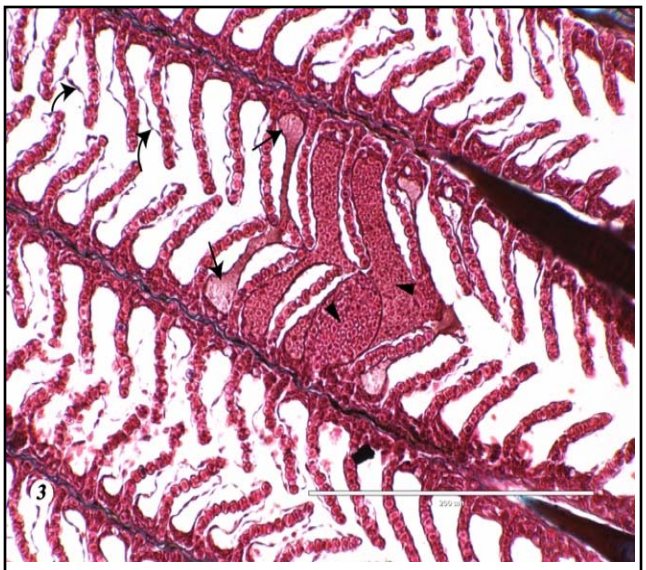

Figure 3. The gill of an SMC fish showing marked dilation of the blood vessels (telengectasia) (arrowheads) and epithelial detachment of the secondary lamellae (curved arrows) causing edema (arrows).

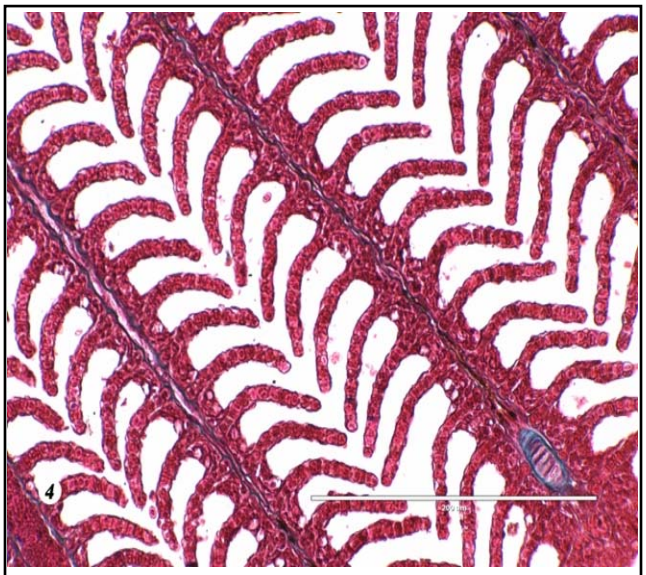

Figure 4. An uninfected gill from the LC population. Note the well defined secondary lamellae, lack of epithelial detachment, edema, vascular dilation and mucous cells.

(Table I). RCs were observed in the gills of both populations where they were seen to occupy an area of the perichondrium beneath the cartilaginous knob that supports the base of the filament as part of the branchial skeleton. The majority of these cells were oriented so that the rodlets faced the lumen of the afferent branchial artery (Figure 5). We did not observe RCs associated with the parasites within either the 1^0 or 2^0 lamellae.

Surprisingly, even though the number of parasites/area in the SMC population differed significantly ($p<0.001$) from the LC population (Table I), the mean number of RCs did not differ ($p>0.05$) between the two populations (Table II). Furthermore, a correlation did not exist between parasite numbers and RC counts in either population (Table III); in the highly impacted SMC fish $r=0.173$ ($p>0.05$) compared to $r=0.095$ ($P>0.05$) in the LC fish from the non-impacted site (Table IV). Furthermore, there was no significant difference in the mean number of RCs/ area between males (55.6 ± 9.9) and females 41.8 ± 5.5) of the SMC population and among the males (50.0 ± 5.5), females (46.6 ± 4.6) and unknowns (37.9 ± 0.85) of the LC population ($p>0.05$) (Table I). When we tested for a correlation between StL and RCs/area we obtained an $r = 0.14$ for the SMC fish and $r= 0.18$ for the LC population (Table IV), indicating that a positive correlation did not exist between these two parameters.

Ultrastructural observations on the gills of both populations showed that the distribution and activity of the rodlet cells was essentially the same. Mature RCs were most numerous. They were seen in the subendothelial layer of the afferent branchial artery where they were most often oriented perpendicular to the luminal space (Figures 5 and 6). Adhering junctions in the form of desmosomes and tight junctions could often be seen at the apices between the RCs and neighboring endothelial cells (Figure 7). On occasion, homocellular junctions in the form of desmosomes existed between adjacent RCs (Figure 8). The apices of the secreting RCs were observed to be protruding between the endothelial cells into the vascular space (Figures 6 and 7). Discharged rodlets were observed occasionally within the vascular space (Figure 9). A small number of spent RCs that had become apoptotic were present within the elements of the perichondrium (Figure 9).

In addition to RCs, intact EGCs were on occasion, present within the sub-vascular tissue of the gills of fish from both populations (Figures 7 and 10). These purported analogs of the mammalian mast cell[15,16] had not degranulated, coinciding with a lack of tissue inflammation or necrosis.

Discussion

Histopathological changes in the gills of fishes have often served as a monitor of environmental stress. The structural impact that we observed in the gills of the SMC fish from the highly contaminated site were consistent with those of others who observed a negative effect of such factors as parasite infestation[13,42,43], changes in water temperature[44], salinity[45] and pH[46]. Pathological changes in the gills have also been reported for fish exposed to petroleum effluents[47] while heavy metal exposure has been found to be especially damaging to gills[32.33]. Typical pathologies that we observed included dilation of blood vessels (telangiectasia), epithelial lifting of the secondary lamellae, hyperplasia of the epithelium in the interlamellar zone

Table I. The mean number of parasites/section in SMC and LC populations of *Fundulus heteroclitus*[1]

Saw Mill Creek[2]		Lemon Creek[3]		
Males	**Females**	**Males**	**Females**	**Unknown**
9.55	37.7	0.1	0.09	0.43
23.9	11.9	0.1	0.11	0.11
32.5	18.6	0.1	0.1	
31.4	32.7	0.13	0.2	
	7.53	0	0	
	8.33	0	0.21	
	18.0	1.6	0.11	
	5.8	.08	0.18	
	18.1		0.5	
			0.1	
19.7 ± 5.9*	17.6 ± 3.37*	0.21 ± 0.12*	0.18 ± 0.05*	0.27 ± 0.2*

[1] One-way ANOVA between populations is significant ($p<0.001$)
[2]T-test comparison between males and females is insignificant ($p>0.05$)
[3]One-way ANOVA between males, females and unknowns is insignificant ($p>0.05$)
* Mean ± SEM

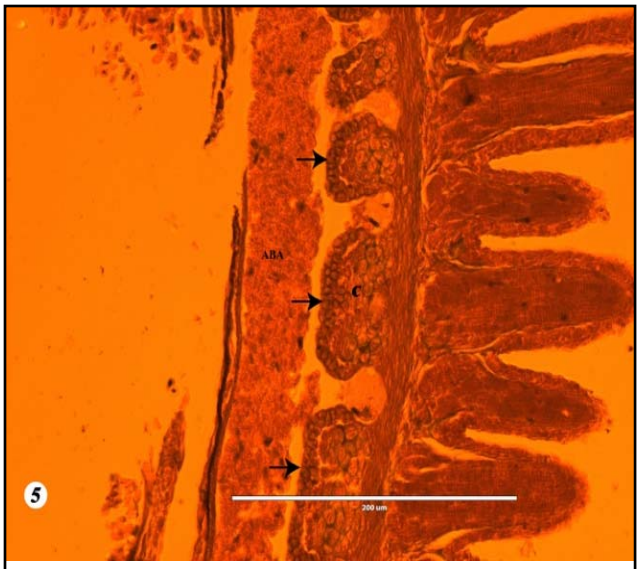

Figure 5. The base of the hemibranch of an LC fish. The rodlet cells (arrows) form clusters within the perichondrium beneath the cartilage (C) of the branchial skeleton and located unneath the afferent branchial artery (ABA).

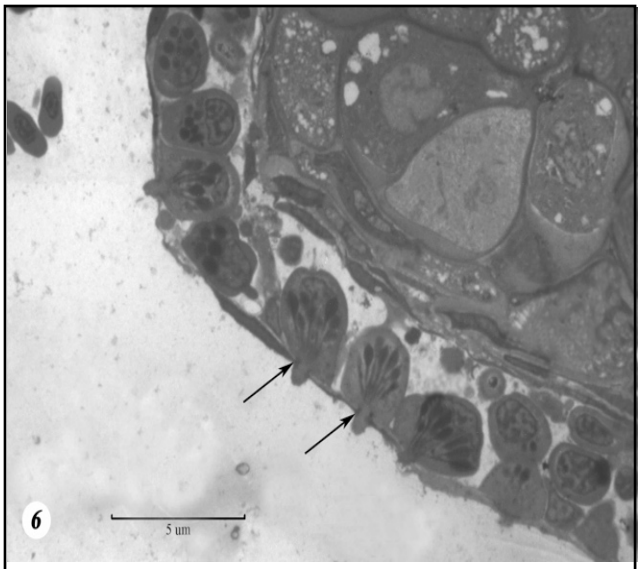

Figure 6. A TEM of the gill from an LC fish. The rodlet cells within the subendothelial layer of the afferent branchial artery are oriented perpendicular to the luminal space. Their apices can be seen protruding between the endothelium. Adhering junctions (arrows) between the rodlet cells and the endothelium are visible.

Table II. The mean number of RCs/area in SMC and LC populations of *Fundulus heteroclitus*[1]				
Saw Mill Creek[2]		Lemon Creek[3]		
Males	Fmales	Males	Females	Unknown
57.7	60.3	56.7	28.6	38.8
39.5	60.3	41.5	40.7	37.1
34.2	33.3	33.8	53.4	
91.0	30.6	63.4	41.7	
	56.6	76.3	47.6	
	41.0	74.1	44.3	
	53.5	40.9	28.1	
	24.4	53.7	75.4	
	15.9		68.8	
			55.2	
55.6 ± 9.9*	41.8 ± 5.5*	50.0 ± 5.5*	46.6 ± 4.6*	37.9 ± 0.85*

[1]One-way ANOVA between populations is insignificant (p>0.05)
[2]T-test comparison between males and females is insignificant (p>0.05)
[3]One-way ANOVA between males, females and unknowns is insignificant (p>0.05)
*Mean ± SEM

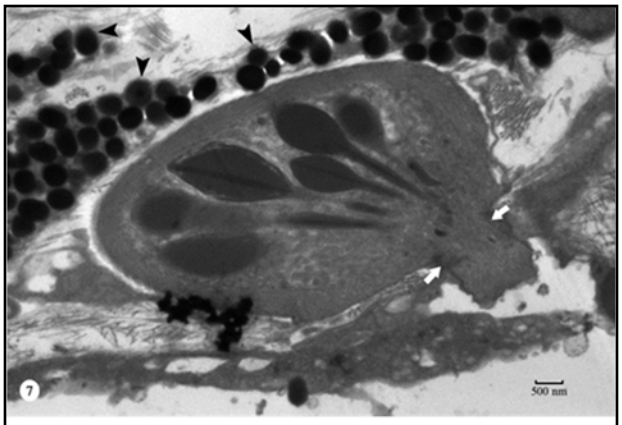

Figure 7. A TEM of mature rodlet cell secreting into the afferent branchial artery of the gill of an SMC fish. Note the adhering junctions (arrows) between the rodlet cell and endothelial cells together with EGCs (arrowheads).

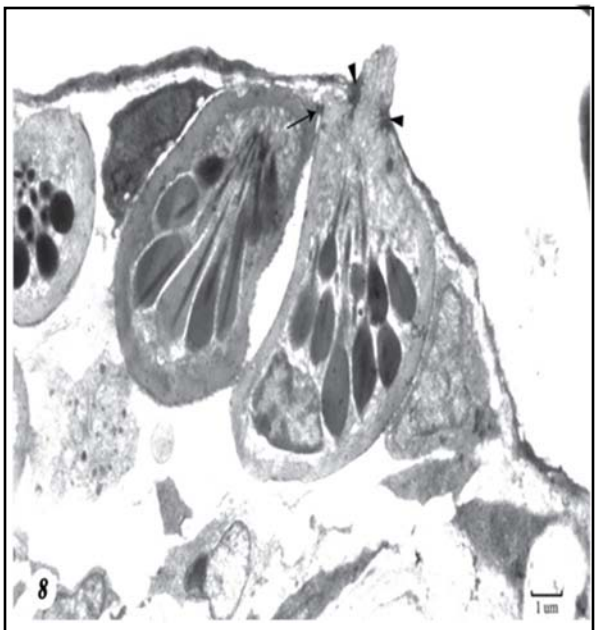

Figure 8. A TEM of the perichondrial region of the base of the gill filament underlying the afferent branchial artery of an LC fish. Adhering junctions in the form of demosomes (arrows) and tight junctions (arrowheads) are seen between the rodlet cells and the rodlet cells and endothelium respectively.

Table III. A correlation between mean parasites/section and RCs/area in SMC and LC populations of *Fundulus heteroclitus*[1]

Saw Mill Creek		Lemon Creek	
Parasites/Sec.	**RCs/Area**	**Parasites/Sec.**	**RCs/Area**
37.7	60.3	0.43	38.8
11.9	60.3	0.1	56.7
18.6	33.3	0.09	28.6
32.7	30.6	0.11	40.7
7.53	56.6	0.1	53.4
9.55	57.7	0.2	41.7
8.33	41.0	0.1	41.5
23.9	39.5	0.1	33.8
18.0	53.5	0	47.6
32.5	34.2	0.13	63.4
5.8	*24.4*	0.21	44.3
31.4	91.0	0	76.3
18.1	15.9	0.11	28.1
		0.11	37.1
19.7 ± 3.1*	46.02 ± 5.43*	0	74.1
		0.18	75.4
		0.5	68.8
		1.6	40.9
		0.08	53.7
		0.1	55.2
		0.21 ± 0.07*	48.3 ± 3.1*
r= 0.1728 p>0.05		r= 0.095 P>0.05	

[1]Data was combined in each population as a statistical difference in RCs/area did not exist (p>0.05) between males and females (SMC) and males, females and unknowns (PB) (Table II)
*Mean ± SEM

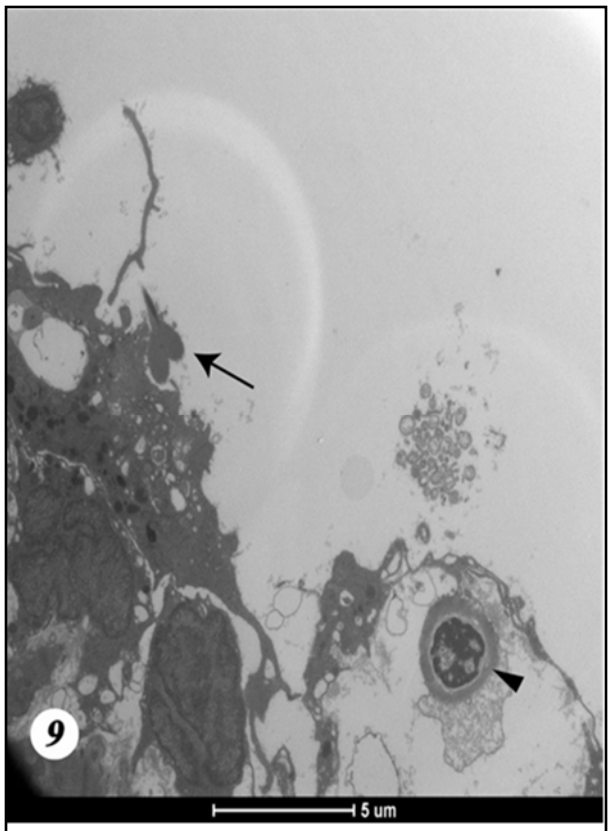

Figure 9. A TEM of the basal portion of a hemibranch from an SMC fish showing discharged rodlets (arrow) and a spent rodlet cell (arrowhead). Note the retracted membrane.

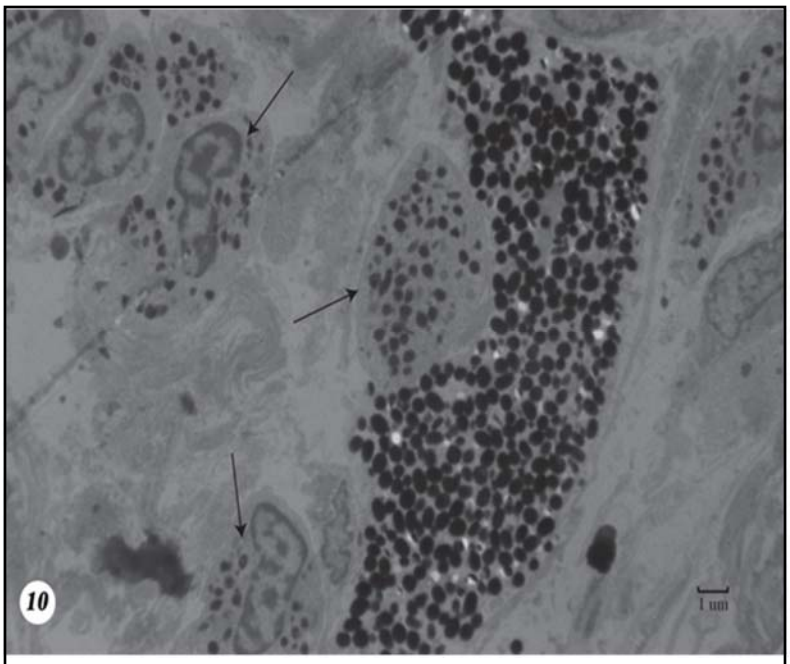

Figure 10. A TEM showing EGCs (arrows) within the subvascular tissue in the gill of an LC fish.

between the secondary lamellae, secondary lamellae fusion, increased melanin deposition and mucous cells and the presence of rodlet cells.

An innumerable number of studies have come forth in recent years that support the recruitment of rodlet cells as effectors in the immune response of various tissues to the physical, chemical and parasite perturbations imposed by the environment[8,20,26,27,48]. As a consequence, the rodlet cell has been viewed by many to serve as a biomarker for evaluating the potential negative impact of the environment on the life of the fish. Studies on the gills of fish exposed to environmental stressors have shown that rodlet cells were recruited and associated with specific histopathologies. Shimada Borges *et al*.[27], observed a statistically greater number of rodlet cells in affected gills of the Nile tilapia, *Oreochromis niloticus* collected from an urban lake contaminated with high levels of phosphate and chlorophyll compared to two other less contaminated sites where the tissue damage was less severe and the rodlet cells were fewer in number. Santos Procopio *et al.*[28], observed a greater number of rodlet cells in the gills of *Prochilodus argenteus* collected from a site that was highly contaminated with heavy metals compared to fish obtained from a low impacted site. The degree of tissue damage was greatest in the former group. Recently, Schultz *et al.*[29], investigating the gills of the Murray Cod, *Maccullochella peeli* suffering from chronic ulcerative dermatopathy, found that there was a greater number of rodlet cells in the damaged gill tissue than in unaffected fish. According to these investigators, the cause was

Table IV. A correlation between StL and Mean RCs/area in SMC and LC populations of *Fundulus heteroclitus*.[1]

Saw Mill Creek		Lemon Creek	
StL (mm)	**RCs/area**	**StL (mm)**	**RCs/area**
69.4	60.3	31.7	38.8
82.8	60.3	35.0	56.7
60.1	33.3	37.8	28.6
52.5	30.6	43.6	40.7
74.0	56.6	56.2	53.4
60.7	57.7	43.3	41.7
60.8	41.0	49.7	41.5
54.8	39.5	31.9	33.8
61.5	53.5	75.4	47.6
73.5	34.2	42.9	63.4
83.9	24.4	55.9	44.3
67.5	91.0	39.3	76.3
86.5	15.9	30.0	28.1
		29.8	37.1
		45.5	74.1
		34.0	75.4
		51.9	68.8
		32.5	40.9
		44.9	53.7
		34.9	55.2
68.3 ± 3.1*	46.02 ± 5.4*	42.3 ± 4.0*	46.2 ± 3.8*
r= 0.14 P>0.05		r= 0.18 P>0.05	

[1] Data was combined in each population as a statistical difference in RCs/area did not exist (P>0.05) between males and females (SMC) and males, females and unknowns (PB) (Table II)
*Mean ± SEM

of unknown origin, presumably a response to a water contaminant. Similar results have been reported in studies of fish exposed to other perturbations affecting gill structure including parasite infection[12,13,49], water-borne contaminants such as the herbicides Propanil[26] and terbuthylazine[50] and osmotic stress[51].

Our results show that in *F. heteroclitus* collected from a highly contaminated habitat (SMC) with high pollutants and having heavy gill infestation with parasites, although histopathological lesions existed in the gills, the number of rodlet cells did not differ compared to the gills of fish collected from a far less impacted site (LC). Furthermore, although the mean number of parasites /section differed significantly between each population, there was no correlation between parasite counts and the number of rodlet cells in either collection. A similar observation was made by Densmore *et al.*[52] who reported that no correlation could be made between the number of rodlet cells and the parasitized tissues of the snakehead, *Channa argus.*

Within each population, the number of rodlet cells did not differ between males and females nor was there a correlation between standard length and rodlet cells/ area. Thus, the rodlet cell number was not a factor of the size of the fish, i.e., the larger fish would be expected to have a greater number of rodlet cells. The fact that we did not observe a difference in rodlet cell counts between the sexes answers a valid question asked by some who suggest that sex hormones could affect the rodlet cell population[28,53] and should be taken into account when making comparisons between the sexes when using rodlet cells as biomarkers. Jordanova *et al.*[53] reported that the number of rodlet cells in the liver of female trout, *Salmo letnica* increased from early to late stages of ovarian maturation, presumably the effect of ovarian sex steroids. Santos Procopio *et al.*[28], observed differences in rodlet cell counts in the gills of male and female curimba, *Prochilodus argenteus* collected from a site highly contaminated with heavy metals. In contrast, our observations show that gender was not a factor that affected rodlet cells, at least in *F. heteroclitus*. Our finding is consistent with that made by Koponen and Myers[48] who observed that the sex of the fish had no apparent effect on the number of RCs in the bream, *Abramis brama* that were chronically exposed to PCBs.

The fact that the number of RCs/area did not differ in the LC fish in comparison to the SMC fish poses an interesting conundrum. Even though the LC fish were exposed to a low impacted environment their RC numbers could have represented an immune mobilization of these cells against an as yet determined physical or chemical contaminant. This would explain why the number of RCs was statistically the same as those in the gills of the fish exposed to high contamination and parasite infestation where the RCs would be expected to be mobilized. One could suggest that even though the RC counts did not differ, the cells of the SMC fish could have been more active as the fish were more immunologically challenged than the LC fish. This was not the case, however. The RCs in both populations did not show any difference in the degree of recruitment and or secretory activity. We never observed RCs to be associated with the parasites in either the primary or secondary lamellae. Therefore, the possibility exists that in the gills of *F. heteroclitus*, RCs may not have been the primary immune effector cell. Instead, this task might have fallen on other effectors such as the EGCs (mast cell

equivalent[15,16,54]) macrophages, granulocytes and non-specific cytotoxic cells[48]. Other than mucous cells, melanocytes and a modicum of EGCs, however, we did not observe any of the other effector cells in appreciable numbers within the gills of either fish. Other studies lend support to this claim. When describing gill histopathologies resulting from exposure to environmental contamination or parasite invasion, other investigators have made no mention of any changes in the RC population although they reported that other immune effector cells such as mucous cells, lymphocytes and EGCs/mast cells had been mobilized[55-59]. Pawert *et al.*[55] observed histopathological changes in the gills of brown trout, *Salmo trutta* and loach, *Barbatula barbatula* exposed to a heavily polluted stream. However, although the gills of each fish were significantly affected, RCs were present in all groups, the controls as well as fish exposed to pollution. The authors made no mention of quantitative differences in these cells between the fish.

In conclusion, the data reported herein strongly support our claim that RCs do not serve as a reliable biomarker, at least in the gills of *F. heteroclitus*. Therefore, the presence of these cells in this tissue should be viewed more cautiously when evaluating the impact of the environment on the health of fishes in general.

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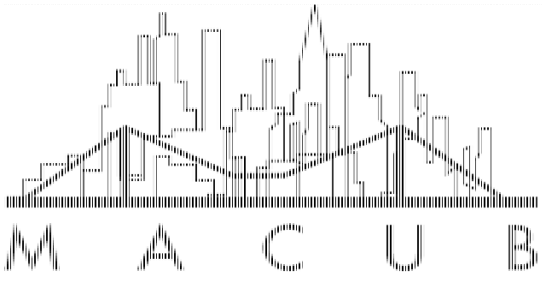

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