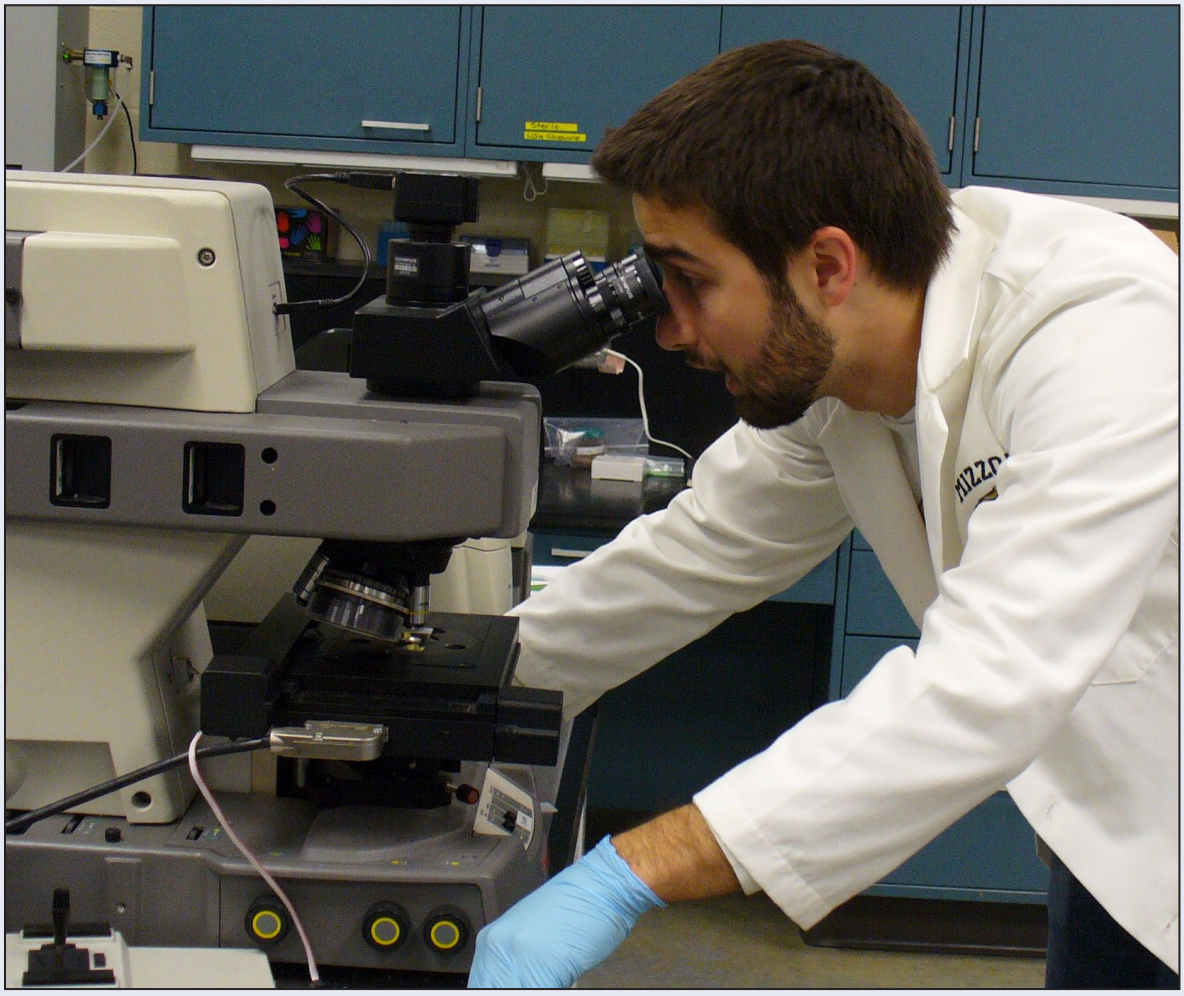
McNair Journal

FALL ■ 2012



McNair Scholars Program

University of Missouri

Cover Photo - Provided by McNair Scholars Program

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RONALD E. McNAIR POST-BACCALAUREATE ACHIEVEMENT PROGRAM

On the Cover: Damir Kolasinac , Department of Biological Engineering.

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Message from the Director

It is with great pride that I introduce this outstanding collection of articles from the 2011-12 participants of the MU McNair Scholars Program. The papers presented here represent the culmination of a year's worth of research and scholarly activity. They reflect the energy, creativity and effort of the scholars, themselves, as well as the careful guidance, support and diligence of their faculty mentors. Six very diverse topics are explored and reported in their entirety within this interdisciplinary journal. While their subject matter and journalistic styles may differ, they, along with the other McNair Scholars listed in this publication, are to be commended for their persistence and dedication to this rigorous undergraduate research experience that will benefit them greatly in their pursuits of graduate studies.

Since 1989, the McNair Program has been a University-wide effort that continues to attract students and faculty mentors from a variety of academic departments and fields of inquiry. Students have had the opportunity to learn about the importance of earning an advanced degree, while gaining the skills and tools that will guide them through their future academic journeys. The program proudly bears the name of astronaut and scientist, Dr. Ronald E. McNair, who died in the Challenger explosion in 1986. His accomplishments and high standards set an outstanding example for these developing scholars.

I am truly honored to be associated with an initiative such as this. So many faculty, staff and administrative members of the MU community have worked to ensure a supportive and cohesive environment that prepares these exceptional students for graduate programs. We are proud to highlight the work of these talented young researchers, in this, the twentieth edition of the **MU McNair Journal**. Our best wishes go out to all of them as they continue to move along their scholastic continuum.

NaTashua Davis, PhD
Director
McNair Scholars Program

The McNair Scholars Program

BACKGROUND

College students who are considering study beyond the baccalaureate level realize their dreams through the McNair Scholars Program at the University of Missouri-Columbia (MU). MU was one of the original fourteen universities selected to develop a program established by the U.S. Department of Education and named for astronaut and Challenger crew member Ronald E. McNair. The purpose of the program is to provide enriching experiences that prepare eligible students for doctoral study.

PROGRAM ELEMENTS

One of the most exciting aspects of the McNair Scholars Program is the opportunity for junior or senior undergraduate students to participate in research experiences. McNair Scholars receive stipends to conduct research and engage in other scholarly activities with faculty mentors from the areas in which they hope to pursue graduate study. These research internships are either for the academic year or for the summer session and are under the supervision of faculty mentors. For academic year internships, students work a minimum of ten hours per week during the fall and winter semesters. Summer interns work full-time for eight weeks.

McNair Scholars also attend professional conferences with their mentors, go to graduate school fairs, prepare for graduate school entrance exams, receive guidance through the graduate school application process and obtain information on securing fellowships, graduate assistantships, and loans. Participants learn about graduate school life, advanced library skills, and effective ways to present their work. At the completion of the research internships at MU, McNair Scholars make formal presentations of their research to faculty and peers at the McNair Scholars Conference and submit papers summarizing their work. Students who participated as juniors the previous year continue in the program during their senior year for graduate school placement and to further develop their skills.

ELIGIBILITY

Participants must meet grade point average standards; be U.S. citizens or permanent residents; and qualify as either a first generation college student with an income level established by the U.S. Department of Education, or a member of a group that is underrepresented in graduate education.

All students who wish to be involved submit an application to the program. A committee composed of faculty members and representatives from both the graduate dean's office and the McNair Scholars Program selects participants and approves faculty mentors. Research internships are offered to those students who are juniors or seniors and are identified as having the greatest potential for pursuing doctoral studies.

Improved Methods Of Cryopreservation Of Biological Specimen Using Geometrically Modified Vials

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Darnell Cage is from St. Louis, Missouri and is majoring in mechanical engineering with a minor in math, aerospace engineering and leadership & public service. He is president of the Mizzou Black Men's Initiative and is involved with the Homecoming Steering Committee, Alpha Omega Theta Christian Fraternity, and Tau Bet Pi and Pi Tau Sigma Engineering Honors Fraternities. Darnell is a Gates Millennium Scholar and a Diversity in Engineering Scholarship recipient. This summer, Darnell interned as the direct assistant to the CEO of Boeing Defense Space & Security in St. Louis. His future plans involve becoming a patent attorney.

ABSTRACT

Cryopreservation is the freezing of biological cells or whole tissues to subzero temperature to facilitate preservation. In cyropreservation, two factors play a significant role in determining the effectiveness of a cyropreservation protocol: uniform temperature distribution and cooling rate. An effective cooling rate is neither too fast nor too slow. If the cooling rate is too slow, cells are at risk of dehydrization and if it is too fast, the cells are at risk of ice crystallization (intracellular ice) which causes expansion of the water molecules within the cell and ultimately damages the integrity of the cell structure. Uniform temperature distribution deals with an effective cooling rate within all confines of the cell so that each aspect of the cell experiences the same cooling rate throughout. In traditional methods of cryopreservation, both of these factors suffered which sparked development of a new, more efficient method. The freezing of a room temperature specimen requires insertion inside vessel filled with liquid nitrogen which is around -210°C under normal atmospheric pressure. In traditional methods the liquid nitrogen overpasses the exterior of the specimen, only allowing for rapid cooling rates for the exterior of the structure with slower cooling rates for the interior. The focus of this experiment was to optimize the design of the process to obtain the best cooling rate and uniformity of temperature distribution. With an improved design that added an addition pathway through the center of the holding wells of the specimen, liquid nitrogen was allowed to overpass the exterior as well as through the center of the specimen. This provided a significant decrease in thermal resistance that generated significant increases in uniform temperature distributions and cooling rates when in comparison to traditional methods. Fine thermocouple was used to send data to a rapid data acquisition system to measure the temperature of the specimen at precise points throughout the cooling and obtain the change in temperature with respect to time. In comparison with numerical simulations, the experimental values obtained held true to expectations. This process is proven to be repeatable and vastly effective.

INTRODUCTION

The goal of the current set of experiments is to evaluate the spatially-dependent cooling rate in geometrically modified vials. Numerical heat transfer simulations of an annular vial geometry show more uniform cooling rates than conventional cylindrical vials. The explanation for the improved uniformity is based on the surface-area-to-volume ratio increase for annular vials compared to the cylindrical vial. Experimental measurements and numerical analysis will be carried out in both cylindrical and annular prototypes to clearly compare the different designs.

The problem is that cylindrical vials exhibit spatially non-

uniform cooling rates. Changing the geometry of the vial by adding a middle channel increases the surface-area-to-volume ratio which should reduce spatial variation. Experiments on these redesigned vials need to be conducted on vial prototypes to validate this claim.

Research subproblems include finding a strategy to calculate eigenvalues for the analytical model, determining which software approach will be most effective in determining a numerical analysis approach, which cooling fluid should be used to increase cooling (liquid nitrogen only, liquid nitrogen/alcohol slush, or other method), and developing a process to build prototypes to successfully simulate small vials

In order to Analyze Subproblems and collect data, numerical and/or analytical simulations that calculate cooling rates across an optimized vial model are needed along with experimental trails that calculate cooling rates between the inner and outer walls, experimental data on prototypes to validate models, and property information for biological materials from the literature. It is hypothesized that a modified cryopreservation vial will lead to more spatially uniform cooling rates. Delimitations include the fact that biological samples will not be directly used.

NOMENCLATURE

Two factors play a significant role in determining the effectiveness of a cyropreservation protocol: uniformity of temperature and cooling rate. If the cooling rate is too slow, cells are at risk of dehydrization. uniformity of temperature deals with an effective cooling rate within all confines of the cell so that the same cooling rate throughout. In traditional methods of cryopreservation, both of these factors suffered which sparked development of a new, more efficient method. The freezing of a room temperature specimen requires insertion inside vessel filled with liquid nitrogen which is around -210°C under normal atmospheric pressure. In traditional methods the liquid nitrogen overpasses the exterior of the specimen, only allowing for rapid cooling rates for the exterior of the structure with slower cooling rates for the interior. The focus of this experiment was to optimize the design of the process to obtain the best cooling rate and uniformity of temperature distribution. With an improved design that added an addition pathway through the center of the holding wells of the specimen, liquid nitrogen was allowed to overpass the exterior as well as through the center of the specimen. This provided a significant decrease in thermal resistance that generated significant increases in uniform temperature distributions and cooling rates when in comparison to traditional methods. Fine thermocouple was used to send data to a rapid data acquisition system to measure the temperature of the specimen at precise points throughout the cooling and obtain the change in temperature with respect to time. In comparison with numerical simulations, the experimental values obtained held true to expectations. This process is proven to be repeatable and vastly effective.

This research is focused on improving the design of vials for cryopreservation. Traditional vials used for cryogenic storage of biological specimens such as blood, cells, etc are generally constructed out of plastic or glass and offer advantages when it comes to cost, transparency, and producibility, but are disadvantaged in their poor conduction of heat transfer and cumbersome structural design. Our numerical heat transfer simulations show this type of design is unsuccessful in two significant factors that determine the effectiveness of cryopreservation protocol: producing uniformity of temperature and rapid cooling rates for certain cases. In this structural design, only specimen that was tangential to the walls of the vial would receive a rapid cooling rate while the inner specimen cooled much slower. To overcome these disadvantages we developed an improved design that added an additional channel through the center of the vials so liquid nitrogen used for the cooling process was allowed to pass through the center of the vial. This provided a significant decrease in thermal resistance from the wall to the inner specimen and nearly doubled the heat transfer area which generated significant increases in uniform temperature distribution and increased cooling rate. Numerical heat transfer simulations for this modification implicated the effectiveness of the design that was subsequently followed by experimental evaluations.

In the experimental evaluations, a 25 micron bead of type E fine bare thermocouple was inserted into the traditional commercial vial and the corresponding location of the new design to measure the temperature at precise points within the vials as it was inserted into liquid nitrogen. A rapid data acquisition system was used to measure the change in temperature with respect to time. The matched numerical simulations and experimental evaluations proved this design to be effective and usable for future cryogenic storage applications. This study will focus on improved methods of preservation of biological specimen specifically through cryopreservation through geometrically modified vials.

This study will focus on improved methods of preservation of biological specimen specifically using geometrically modified cryovials. A cryovial is a container that holds biological specimens while they are cryogenically preserved. Our study focuses on preserving biological material at the cellular level. What we learn about the cryopreservation process will allow us to preserve larger forms of biological matter including tissues and organs. A review focuses on describing methods of preservation and its needs and contributions to society including preserving biological matter including human oocyte, umbilical cord blood, organs, and tissues. This review will discuss the importance of preservation and its role in society as well as the different methods of preservation currently in use and under various stages of development.

Preservation

Important biological material targeted for preservation are organs, tissues, cells, and blood. Ideally, this would be done at low temperatures to maintain the integrity of the biological specimen. Unfortunately, the freezing process generates ice crystals that damage the viable biological materials. Thus, successful low temperature storage remains elusive.

For organs, “preservation aims to provide a viable graft with primary function post-transplant”. Organs to be preserved are the heart, liver, and kidney [8]. This issue has increased in importance since the rise in demand for organs has begun to exceed the supply of organs from young, healthy donors [6]. Consequently, there has been a progression in the acceptance of older and marginal donors, defined as non-heart beating, which have suffered from additional warm ischemic injury therefore having

higher primary nonfunction and delayed graft function rates than that of younger donors [5]. If organ donors do not find a match for their organs in the time they remain viable, the organ must be discarded, going to waste. Not only is this the case in organ donations, but also for tissues and cells.

Preservation of human blood vessel tissue at lower temperatures can help to bypass surgery and peripheral vascular reconstruction procedure [7]. Preservation of human oocytes helps to be able to maintain female fertility by having the ability to preserve a fraction of a females limited amount of eggs in situations that would otherwise destroy or cause them to go to waste such as chemotherapy and infertility [2]. Preservation of umbilical cord blood could aid in the development and implementation of stem cell therapies which may help sustain the quality of life in newborns when they age [4]. However, these preservations will not become a clinical practice until the availability of a reliable form of long term storage [10].

Methods of Preservation

Static Cold Storage

Most organ preservation centers today use static cold storage (CS) as their means of preserving organs and has become the preferred method across the practice. According to Maathuis et. al, "simple cold storage starts with a rapid vascular washout to allow cooling of the organ, removal of blood components, and equilibrate the CS [cold storage] solution with the tissue". This method uses hypothermia to slow down metabolism and catabolic enzymes in helping to maintain the integrity of cells. However, this leads to harmful side effects including "cell swelling, acidosis, and the production of radical oxygen species upon reperfusion". These harmful side effects are combatted by adding CS solution containing specific compounds. This is known as the solution effect and it also seeks to prevent cell swelling, the production of reactive oxygen species (ROS) which can result in cell death and damage to lipids, nucleic acids, and proteins which are vital to cell survival. CS was developed in a time when more organ donors were younger and had a stronger quality of organs, but an implantation on more stringent donor criteria has led to this method reaching its limits [5]

Vitrification

Brian Wowk defines vitrification in "Thermodynamic aspects of vitrification" as "a process in which a liquid begins to behave as a solid during cooling without any substantial change in molecular arrangement or thermodynamic state variables [11]. This process focuses on bypassing the solid state of water which forms ice crystals to the "solid liquid", or glass state. This is done by cooling the temperature far below their freezing point to the glass transition temperature. Some elements, including water, are able to avoid the crystallization of their molecules if they are exposed to rapid cooling rate and achieving a rapid cooling rate increases the likelihood of this occurring. In the glass state, molecules have properties similar to that of a solid and a disordered molecular structure as if they "were frozen in time". This process of achieving a glass state by freezing is known as vitrification and is important because it minimizes the amount of crystallization and thus expansion of water in cells which ultimately minimizes the damage to the structural integrity.

Cryopreservation

David E Pegg in Methods in Molecular Biology, vol. 368 defines cryopreservation as "the use of very low temperatures to preserve structurally intact living cells and tissue" [9]. This is typically done by placing the specimen in a cryo-storage device, such as a vial or tube, and plunging it into liquid nitrogen. There are commonly used techniques of cryopreservation: cryopreservation by slow cooling and cryopreservation by vitrification. Cells are primarily composed of water and are intricately distributed throughout cell structure bounded by the cell membrane or cell wall. Typically during freezing, water goes from liquid to solid. Water, unlike most substances, expands under freezing by a rearranging of molecules. An expansion in volume of water in a cell designed to only hold as much volume of water in the liquid state causes damage to the structural integrity of the cell. At an organ or tissue level, this become deleterious as this overdistends luminal spaces [3] and runs the risk of "loss of both smooth muscle contractility and endothelial function" which are vital to homeostasis [10]. This is the primary concern for both cryopreservation techniques.

Freezing

Contrary to vitrification, slow cooling does not seek to stop the formation of ice crystals from freezing water, but rather to eliminate or minimize the total amount of water within the cell. This is done by introducing a cryoprotectant into the cell until it reaches its final equilibrium volume. The amount of cryoprotectant and water needed is determined by the permeability parameters of the cell and the concentration of "impermeant solutes in the solution". The introduction of cryoprotectants to cells can, however, be risky as most cryoprotectants have a level of toxicity foreign and deleterious to the cell. Therefore, the cryoprotectant introduction process must be optimized to be generated at temperatures where the cryoprotectants toxicity is no longer deleterious to the cell [9]. For large oragans, vitrification is challenged by heat and mass transfer limitations, but can be simplified to induce the freezing of tissue and cells [3]. This method is still currently under testing and no perfect process has been created [9].

Dehydration

In an experiment conducted by Elmoazzen, it was determined that mouse sperm can be preserved by drying it "under a stream of nitrogen gas at ambient temperature and stored…". Once rehydrated and performing intracytoplasmic sperm injection after being stored at 4 degrees Celsius, the sperm still maintained all capabilities. This includes fertilization, "… embryo development in vitro to the blastocyst stage in vitro…" and, "… development in vivo to day 15 after the embryo transfer into foster mothers…". The sperm was stored for 3 months and it was demonstrated that they could be stored at -20 and -80 degrees Celsius without deterioration for up to 5 months. Specimens must be stored in vacuum sealed Mylar packages to maintain dehydration and preservation. [1]

It can be logically concluded from the prior stated information that there is a need for preservation of biological specimen to assist in society, especially in the areas of female fertility and organ and tissue donation effectiveness. Current methods, mainly cold storage, are used to be able to preserve the organs for as long as possible, but clearly there is a need for an improved method. Scientists are currently researching different methods, but have taken the lead with cryopreservation

because of its potential and feasibility. Issues currently faced in developing a reliable method of cryopreservation are the challenge of overcoming intracellular ice. Methods such as vitrification and freezing with cryoprotectants are being studied with hope to be able to either increase cooling rate to reach glass temperatures or develop a cooling method that precisely introduces the needed amount of cryoprotectant into the cell at the correct temperature. Our study focuses on these issues by developing an improved method of cryopreservation that increases cooling rates through process and geometrically modified cryo-storage devices.

METHODS

The goal of this project is to improve the cooling rates of cryopreservation of biological specimen using geometrically modified vials. The approach to this process involves an experimental and analytical piece. In previous designs of the cryovials, the upper part of the system consisted of a syringe housing fixed over the top of the vial cap with another cap on the opposing end of the syringe to keep the added center channel in alignment. The new design has a channel which passed through the center of the vial to allow for the passage of liquid nitrogen. A second system was constructed with identical criteria, but with the center channel blocked off. This allowed for a more accurate comparison between the traditional and new designs due to the similar specifications. In the current redesign, we decided to abandon the traditional vials and construct new ones from syringes. As an upscale prototype, we used a 20 mL and a 10 mL syringe cut to the same length and fitted the 10mL syringe inside of it shown in Figure 1. A third design adds a plunger to the center channel of the new vial design shown in Figure 2 and Figure 3.

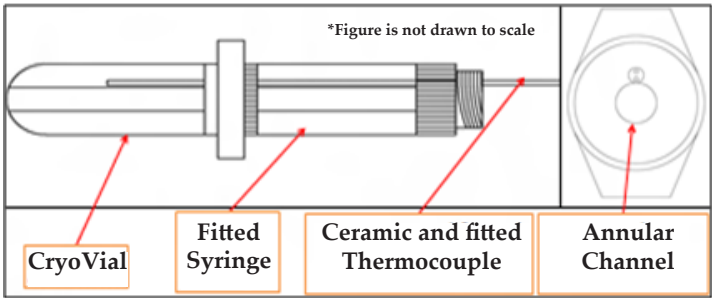

Figure 1. Vial redesign with annular channel

Figure 2. Front view of new plunger vial design

Figure 3. Top view of new plunger vial design

Experimental Procedure

The experiments were conducted by placing a thermocouple with a 25 micro-meter bead at various locations in the prototypes. The thermocouple was threaded through a ceramic sleeve with a ¼ inch outer diameter and 1/64 inch inner diameter. A plunger was fitted inside the inner channel created by to increase the acceleration of the liquid nitrogen through the channel in effort to increase the cooling rate. The ceramic sleeve containing the thermocouple was fit through a hole drilled in the top of the newly designed system 5 millimeters away from the center line of the vial in such a way that it could move in measured increments vertically to measure temperatures at several different positions in both designs. The vials were then plunged into liquid nitrogen while a high-speed data acquisition system measured the transient thermocouple temperature response. Each trial was performed with water and MVF being the observed specimen. The measurement results are compared with the model responses.

Experimental Data Collection

Data was collected via a data acquisition system which takes voltage measurements of the thermocouple. After extrapolating the data from the trials with the high-speed data acquisition system, the data was fitted into a Microsoft Excel spreadsheet, measure the DC wattage output 500 times per second. We then derived the conversion equation for DC wattage to temperature in Celsius from the Omega Engineering's reference for type E thermocouple in order to obtain the temperature. After the temperature data for the vial was obtained, we then obtained the room temperature data using the same system so that that value could be deducted from our temperature values for the vials. We then derived the cooling rate by using the 5 point derivative method with the data that was acquired. All the data was then fit and plotted on using the chart and plotting functions of Microsoft Excel.

Experimental Rationale

Experimental analysis was chosen due to its practicality. One way to observe whether or not the cooling rate has increased given a new vial is to take it through the experimental procedures and observe the temperature and cooling rate. This is beneficial because doing an experiment allowed for observance of the physical occurrences, therefore, allowing us to capture "real data". Some drawbacks include the following: the models can become very expensive, models tend to break or wear down enough to replace over time, and experiments and building models become time consuming.

Analytical Procedure

In order to find an optimal design for the vial prototypes, an analytical approach will also be taken. MATLab will be used to solve the governing heat equation for radial heat transfer:

$$\frac{\partial^2 T}{\partial r^2} + \left(\frac{1}{r}\right)\frac{\partial T}{\partial r} = \left(\frac{1}{\alpha}\right)\frac{\partial T}{\partial t} \quad \text{[Eq. (1)]}$$

Boundary conditions (B.C.) are as follows:

$$-k * \frac{\partial T}{\partial r} = h_1(T_0 - T)\ @r = r_1 \quad \text{[B.C. 1]}$$

$$-k * \frac{\partial T}{\partial r} = h_2(T_0 - T)\ @r = r_2 \quad \text{[B.C. 2]}$$

The following initial condition (I.C.) was used

$$T=T_i \ @\ t=0 \quad \text{[I.C. 1]}$$

A series of m-files will be written to incorporate various values of radii and lengths and solve for λ a non-dimensional representation of temperature was defined as:

$$\theta = \frac{T - T_0}{T_i - T_0}$$

where T is the measured temperature, T_0 is the temperature of the Liquid Nitrogen (LN), and T_i is the initial temperature of the specimen.

Analytical Model Rationale

The rationale behind the analytical solution is to use heat transfer approaches to determine the rates of cooling experienced by the vial. This allows for us to create a theoretically optimal design of the vials. In solving the above equation, the solution is:

$$\theta = \sum_{n=1}^{\infty} E_n \exp(-\lambda_n^2 \tau) * [-f(\lambda_n, Bi_1) * J_0(\lambda_n R) + Y_0(\lambda_n R)] \text{[Eq. (2)]}$$

In order to calculate the solution, we must find the value of l_n which is given by,

$$\lambda = Bi_2\left(\frac{-f(\lambda,Bi_1)*J_0(\lambda*LR)+E*Y_0(\lambda*LR)}{-f(\lambda,Bi_1)*J_1(\lambda*LR)+Y_1(\lambda*LR)}\right) \text{ [Eq. (3)]}$$

Once the values of lambda are found, we can then derive the overall solution.

How to find roots

Roots were found by using the Bisection Method and reordering Equation 3, subtracting lambda from both sides to make the equation equal to zero. The initial requirements are that we have an initial bound [a, b] on the root, that is, f(a) and f(b) have opposite signs.

Benefits

There are several benefits to the analytical approach. Having an analytical solution allows for the varying of different parameters and the predicting of their effects on the model. It saves time from a "guess and check" experimental method and can be mathematically proven. Some drawbacks are that in order to get the model, we have to make a lot of assumptions in our heat transfer analysis and coding can be frustrating and time consuming.

Integration of Models

In the final analysis, parametric studies will be conducted to obtain an optimal design using the analytic model. Different design parameters will change, allowing for observation and analysis their effects on the model to fit design criteria. Models generated will be compared and contrasted to other configurations. There are several things we currently don't know well including the heat transfer coefficient and if phage change analysis is present, the model does not capture it. For model validation, we will have a "calibration check" which will be done by comparing its results to experiments and modifying the model in such a way that we get accurate results. In the end, we will use integration and studies to derive an optimal design for the vials.

RESULTS

Experimental Results

To begin, three vial designs were selected, the plunger design, the annular design, and the traditional cylindrical design. Two experiments were performed: one taking data at just one point inside the specimen and the other using a design to measure temperature at the inner and outer walls and the middle of the specimen for each of the three types of vials. Two trials were conducted for experiment 1 and one trial was conducted for experiment 2. The data was collected using a digital multimeter and a data acquisition machine. Voltage readings of the specimen were taken using 0.001m type E thermocouple. The first set of results shown in Figure 1, Figure 2, and Figure 3 show the Temperature vs. Time for each configuration. Figure 4 and Figure 5 show the first set of experiments for trials 1 and 2 respectively and Figure 6 shows the second set.

The data shows that the annular vial is a significant improvement over the traditional and plunger vials. It is performing at a much higher cooling rate than traditional vials, meaning specimen reaches cryogenic temperatures more rapidly. Uniformity of the cooling during the process has also proven to be more thorough throughout the specimen which eliminates risks mentions prior of intracellular and extracellular ice formations which dehydrate the cell.

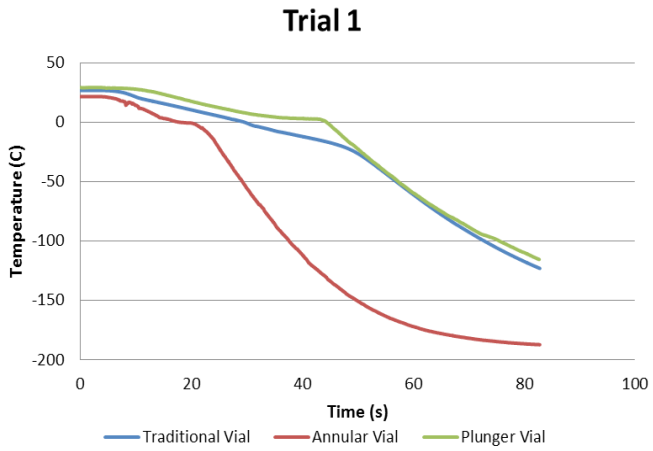

Figure 4. Temperature vs. Time plot for Trial 1 of experiment 1

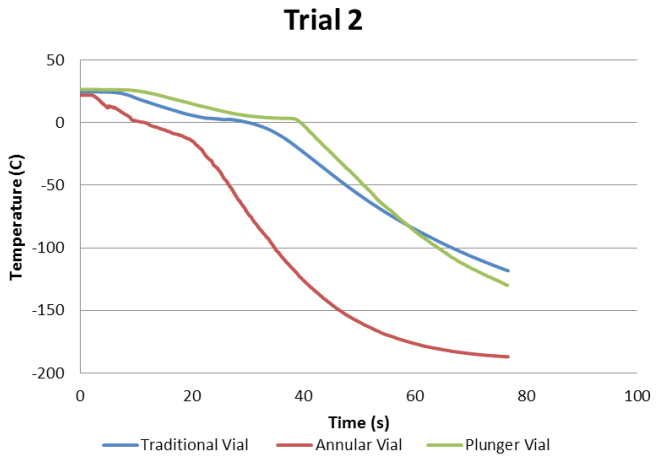

Figure 5. Temperature vs. Time plot for Trial 2 of experiment 1

These temperature plots from both tirals show that the annular vial reaches a steady-state cryogenic temperature (about -196 degrees Celsius) much sooner than the other tested vials. This helps keep the cell from dehydrating from the formation of ice crystals and approaches super-fast cooling.

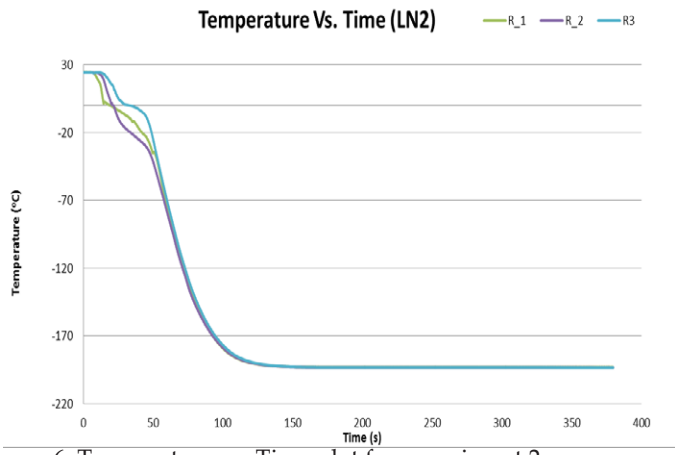

Figure 6. Temperature vs. Time plot for experiment 2

The temperature plot taking at the inner and outer radii and at the middle of the specimen show relative uniformity of cooling throughout the process until the specimen reaches a steady-state. This means that the specimen is cooling simultaneously, helping to preserve the structural integrity of the specimen. Data was then used to calculate the cooling rate for each trial of each experiment and each vial configuration in units of degrees Celsius per second. This was to show how fast the specimen was cooling to see if ultra-fast cooling was achieved. A 200 point moving average was used to eliminate noise in the data plots. Results from this data set are shown in Figure 7 through Figure 12 consecutively. Figure 7 and Figure 8 show the results in plots of cooling rate vs. time of trials 1 and 2 of experiment 1 respectively. Figure 9 shows the results from experiment 2.

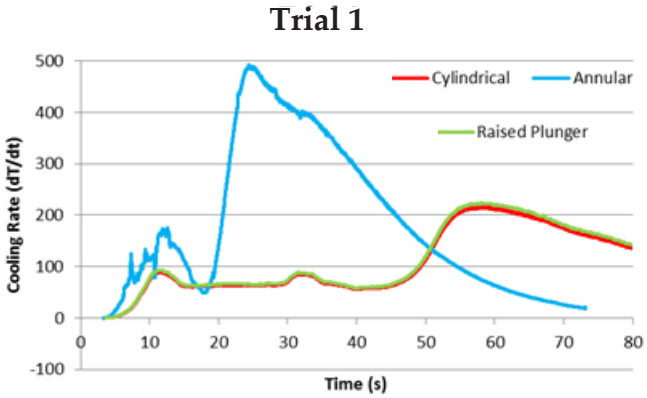

Figure 7. Cooling Rate vs. Time plot for Trial 1 of experiment 1

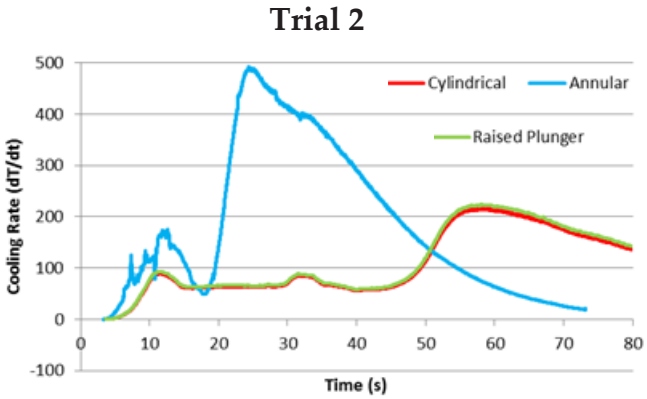

Figure 8. Cooling Rate vs. Time plot for Trial 2 of experiment 1

The cooling rate plots in Figure 7 and Figure 8 demonstrate that the annular vials show a higher cooling rate which occurs much sooner than cooling rate of the other tested vials. Close observance reveals a dip which seperate the plot into two "humps". This occurs during the phase change of water from liquid to solid where the cooling rate and density values change, allowing for higher cooling rates. This, again, shows progress towards approaching super-fast cooling.

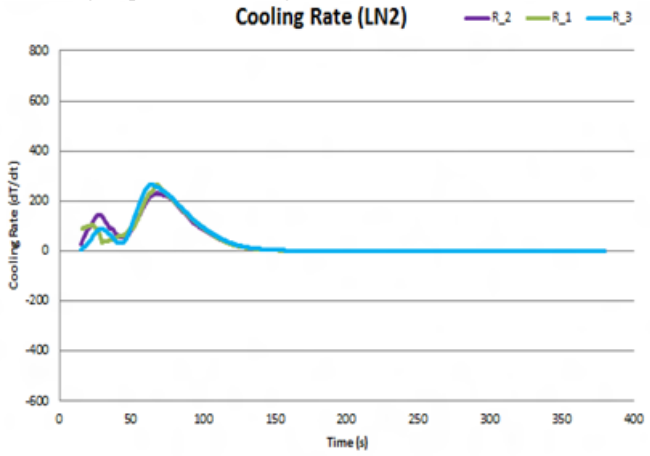

Figure 9. Cooling Rate vs. Time plot for experiment 2

Experiments conducted to produce the prior plot demonstrates the uniformity of the cooling rate throughout the specimen at different radii within the annular vial as seen in Figure 9. This helps to ensure that the cooling process is thorough and helps to maintain the structural integrity of the cell.

The following plots in Figure 10, Figure 11, and Figure 12 show the cooling rate versus temperature of each trial of each experiment. Figure 10 and Figure 11 show the results of experiment 1, trial 1 and 2 respectively. Figure 12 shows the results of experiment 2.

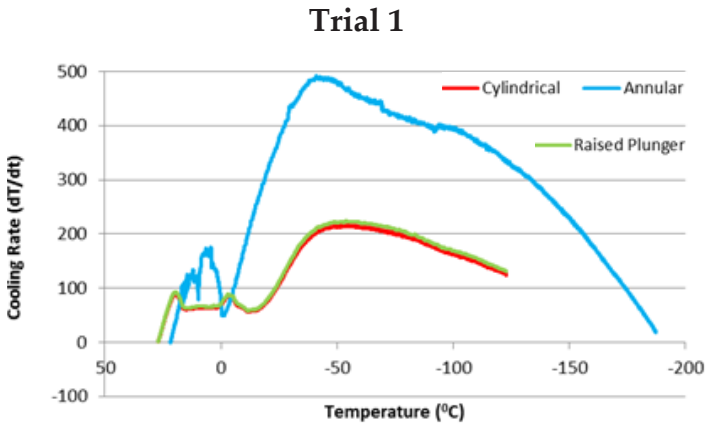

Figure 10. Cooling Rate vs. Temperature plot for trial 1 of experiment 1

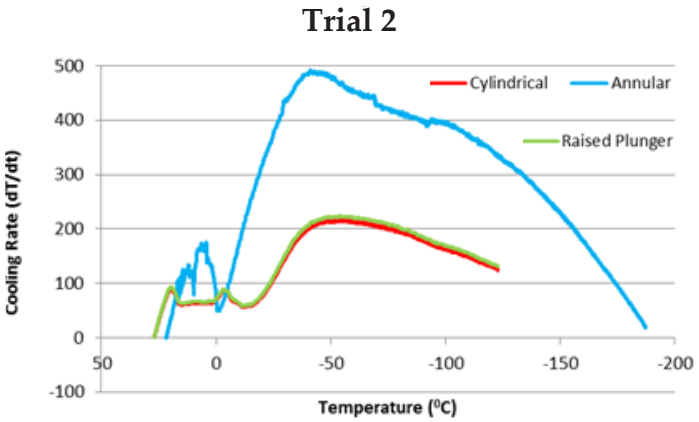

Figure 11. Cooling Rate vs. Temperature plot for trial 2 of experiment 1

As mentioned prior, the behavior of the two "humps" can be explained. In the plots above, in Figure 10 and Figure 11, it shows that at almost exactly zero degrees Celsius, we see a drop in cooling rate due to the latent heat of formation for ice followed by a spike in cooling rate due to the reduced heat capacity and density of the newly formed ice. Still, it shows significant performance in comparison to the other two tested vials.

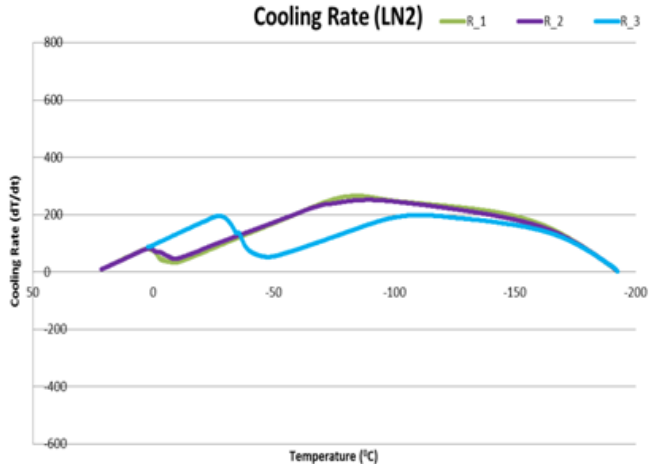

Figure 12. Cooling Rate vs. Temperature plot for experiment 2

Analytical Results

Figure 10 shows the results to date of the analytical experiment. Root finding results, shown in Figure 13, are needed to solve for lambda values for the analytical solution. These roots have been successfully found using the bisection method and experimental plan mention prior in the methods section.

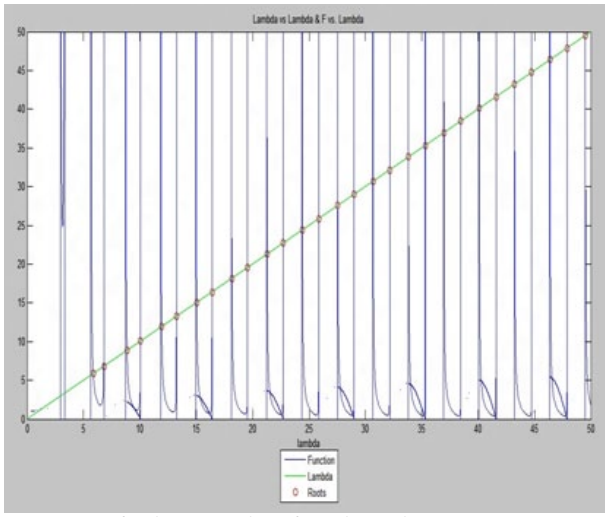

Figure 13. Root finding results of analytical experiment

Numerical Simulations

Numerical simulations were performed to test the validity of the annular vial design as well, the results of which can be found in Figure 14 through Figure 17. Figure 14 and Figure 16 show the results for the traditional cylindrical vial at 0 and 50 seconds respectively, while Figure 16 & 17 show the results of the annular vial at 0 and 50 seconds respectively.

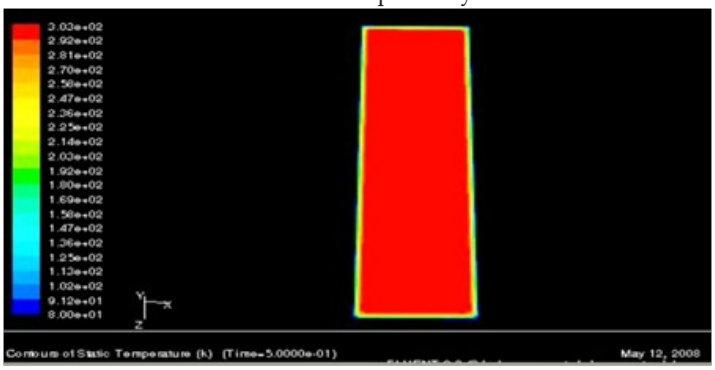

Figure 14. Numerical Simulation results for traditional vial at zero seconds.

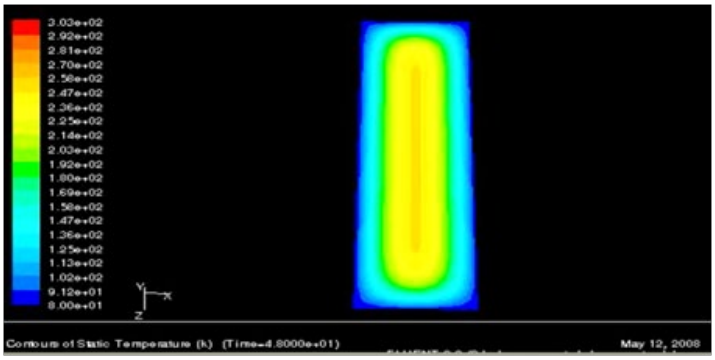

Figure 15. Numerical Simulation results for traditional vial at 50 seconds.

Figure 15 shows that after 50 seconds, the other wall of the vial begins to reach cryogenic temperatures, but the inner specimen is still relatively warm. This leads to extracellular and intracellular ice as mentioned before and ultimately leads to a compromised structural integrity of the specimen, rendering it useless.

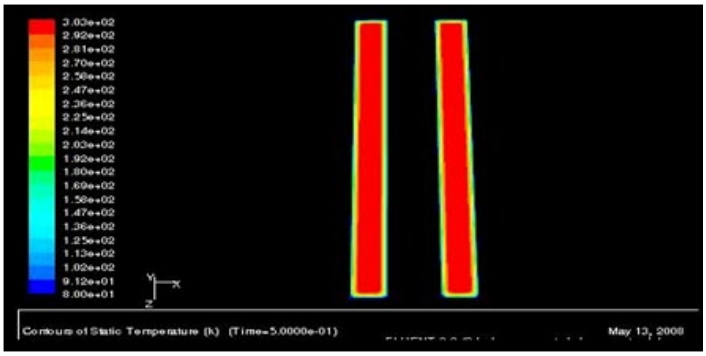

Figure 16. Numerical Simulation results for annular vial at zero seconds.

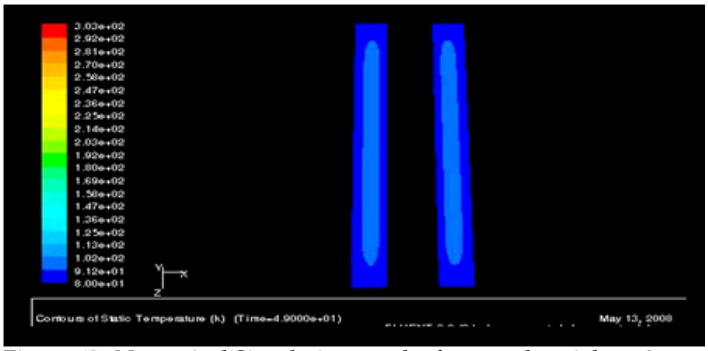

Figure 17. Numerical Simulation results for annular vial at 50 seconds.

Figure 17, in contrast to Figure 15, shows that after 50 seconds, the cooling within the specimen is relatively uniform and much of the specimen is at or approaching cryogenic temperatures. The increased surface area-to-liquid nitrogen ration improves cooling rates and uniformity

Analysis

From the results, it can be seen that the new annular design shows much improvement over the traditional and plunger vials. Higher cooling rates were achieved as well as time to cryogenic temperatures reduced. The results from experiment 2 show how the annular vial design offers near-uniform results in cooling rate and temperature versus time. Interesting are the two spikes in the cooling rate plots. This has been determined to be due to the change in density and heat capacity of water after the phase change from liquid to solid. Analytical results imply that one can successfully solve the analytical solution using MATLab and developing a program to replicate experimental data. Numerical simulations also verify that the annular design can achieve cryogenic temperatures sooner as well as uniformity in temperature distribution.

CONCLUSIONS

In the culmination of these experiments, it can be logically concluded that numerical heat transfer simulations of an annular vial geometry show more uniform cooling rates over conventional cylindrical vials based on the surface-area-to-volume ratio increase for annular vials compared to the cylindrical vial. The addition of a plunging effect effectively decreased the desired cooling rate. It can also be concluded that an analytical model can be comprised to successfully predict cooling rates in our newly designed annular vials. The process has proven to be repeatable and effective.

FUTURE RESEARCH

In the future, there will be a continuance in solving the analytical solution for the model and programming in MATLab. This will be followed by model integration which will compare the experimental data with the model results. In order for this to be effective a "calibration check" which will be done by comparing analytical results to experiments and modifying the model in such a way that accurate results can be achieved must be developed.

ACKNOWLEDGMENTS

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Photoacoustic Detection of *Escherichia coli* Bacteria Cells for Screening of Septicemia

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ABSTRACT

Escherichia coli (E. coli) is the most common gram-negative bacteria to cause septicemia, which occurs when an open wound is exposed to harmful bacteria that then circulates around the body aggregating in different organs, causing more serious issues and eliciting a reciprocating effect. Every year, severe sepsis strikes approximately 750,000 Americans, and an estimated 28 to 50 percent of these people have the potential to die. The current methods for diagnosis and treatment include extensive testing on bodily fluids and administering broad-spectrum antibiotics until a specific strain of bacteria is identified up to five days later. The goal of this research is to isolate and detect circulating bacteria cells in the blood of patients with sepsis within hours, as opposed to days. We will use *E. coli* as a model system in this study. The photoacoustic two phase flowmeter developed by our lab, adapted from detecting circulating melanoma cells, allows us to detect *E. coli* cells. These cells are tagged with antibodies conjugated with dyed microspheres that absorb in the visible spectrum. These dyed microspheres serve to provide optical absorption in an otherwise colorless bacteria cell. The system uses a rapid pulsing laser firing onto absorbing particles that undergo thermoelastic expansion resulting in pressure waves that are then detected by an acoustic sensor. Later, we collect the samples and image them to confirm the *E. coli* detection. The results show that we can tag and detect *E.coli* cells using fluorescence and photoacoustics. Further testing must be done to replicate and improve these results. This method in practical application will hasten the treatment process of patients once they reach the emergency room. The identification of *E. coli* in the blood through a simple blood test in a matter of hours will eliminate the necessity for the non-specific antibiotics and reduce the number of deaths each year resulting from sepsis.

INTRODUCTION

Septicemia generally occurs when an open wound is exposed to harmful bacteria. The bacteria then circulate through the body aggregating in different organs causing infection and sometimes more serious issues throughout. *Escherichia coli (E. coli)* are the most common gram-negative bacteria to cause septicemia. Every year, severe sepsis strikes approximately 750,000 Americans, and an estimated 28 to 50 percent of these people have the potential to die. Currently, in order to diagnose and treat sepsis, one must succumb to extensive testing on bodily fluids and be administered broad-spectrum antibiotics. It is not until three to five day later when a specific strain of bacteria is identified and more specific treatment can be administered.

This project proposes to explore the photoacoustic detection

of *Escherichia coli* bacteria cells for screening of septicemia. The goal of this research is to more quickly detect *E. coli* in the blood of patients with sepsis through the applications of cell tagging and laser optics technology.

We hypothesize that the proper tagging of the *E. coli* cells will allow us to fluoresce and detect them in our Photoacoustic Detection System. We hypothesize that this will be especially true with *E. coli* cells that are tagged with two different nanoparticles.

LITERATURE REVIEW

Sepsis is defined as the systemic response to infection caused by the presence of microorganisms or their toxins in the blood [1]. Septicemia is the leading cause of death in critically ill patients in the United States. It affects 750,000 people per year and more than 210,000 of these people die [2, 3]. Many studies have been carried out attempting to pin point the exact causes of septicemia and differentiate its effects from the effects of other illnesses that manifest similar inflammatory responses [1]. The issue with detection of sepsis is that it can take a number of days to culture a blood sample from a patient, who in the meantime, is given a broad spectrum of antibiotics. Also, the numbers of bacteria found in the blood sample could be improperly calculated if growth has temporarily subsided due to administering drugs before the blood sample was drawn [4].

Methods for the direct detection of *E. coli* have been developing since the 1970's. Some of the more traditional methods of detection include the plate-culture method, DNA hybridization, enzyme linked immunosorbent assay (ELIZA), radioimmunoassay (RIA) systems, and polymerase chain reaction (PCR) assays [9, 10, 11, 12]. Studies have continued attempting to decrease the testing time, the amount of skill and involvement needed to create sensitive systems and achieve accurate results, and to increase accuracy [9, 12]. A study in 1993 explored the development of a fluoroimmunoassay in which "fluorescein isothiocyanate (FITC) conjugated monoclonal anti-Escherichia coli antibody was immobilized onto [bacteria magnetic particles (BMPs)] using a heterobifunctional reagent, N-succinimdyl 3-(2-pyridyldithio)priopionate (SPDP)" [10] and used to detect and remove *E. coli* cells. This study measured the relationship between the fluorescence intensity and the cell concentration after a magnetic field was applied to the mixture of FITC conjugated antibodies, BMPs, and *E. coli* cells. They found that there was, in fact, an indirect correlation between the two with a fluorescence intensity decrease of 78% in the presence of *E. coli* in the 10^3 cells/mL range, while there was no decrease after a 15-minute incubation in the absence of *E. coli*. Although it was successful, this study does not address the ability of the system to adapt to the complexities involved with detection of *E. coli* in samples of blood. The limit of detectable *E. coli*, however, was 10^2 cells/mL, which is, in fact an improvement on earlier methods.

A 1998 study used two multiplex PCR assays to detect STEC in fecal cultures from patients with hemolytic-uremic syndrome and bloody diarrhea. "Assay 1 detects the presence of stx_1, stx_2, *eaeA*, and EHEC *hlyA*, generating PCR products of distinct sizes which are easily distinguished after agarose gel electrophoresis….Multiplex PCR assay 2 detects the presence of genes involved in the biosynthesis of serogroup O111 and O157 O antigen," [13]. The results of this study proved to be successful at detecting the different STEC organisms in the samples and had a relatively decent amount of sensitivity. However, the accuracy of the PCR assays decreased as the number of organisms in the samples decreased. Still, the use of PCR assays in general has been constantly repeated because of the fact that it has one of the highest rates of validity.

A new standard initiated by the American Association of Blood Banks (AABB) in the early 2000s led to an increase in studies for screening for bacterial contaminations in platelet concentrates (PCs) in an attempt to minimize the amounts of bacterial infections resulting from transfusion. A study in 2006 compared three popular rapid detection methods for different bacterial detection simulating actual conditions of the blood [14]. Not surprisingly, two of the three methods included aforementioned designs. This study compared fluorescence-activated cell sorting (FACS) analysis, 16S RNA in-house nucleic acid testing (NAT), and a solid-phase scanning cytometer (optimized scansystem, Hemosystem). The PCs were spiked with four transfusion relevant bacteria: *Staphylococcus aureus, Bacillus cereus, Klebsielle pneumonia,* and *Escherichia coli*, and subsequently tested by each method in replicates of 10. True to tradition, the NAT analysis, similar to other PCR assays, was the only method able to successfully detect all of the different bacterial strains at points where the samples were collected. The other methods had some difficulty confirming bacterial presence at the earlier collection times [14]. Interestingly enough, the optimized scansystem, with the use of general principles of cytometry, is the most resembling of photoacoustic detection, yet had the worst record of presence reporting, particularly with *E. coli*. It has been hypothesized in this study that it could have been related to the efficiency in the staining process of the cells, where uptake can differ from one strain to another.

An interesting approach was explored in a study in 2009 that measured the expression of the CD64 surface neutrophil—a type of white blood cell designed to fight infections—to predict if there was a diagnosis of infection in blood cultures from 109 adult patients with alleged bacterial infections. If there was a CD64 index of ≤ 1.19, the patient was predicted to have no bacterial growth results. If the index was ◎1.19, the patient was predicted to have been positively diagnosed with a bacterial infection. Impressive positive and negative predictive values of 89.8% and 94% respectively, show the value in this method of detection, even without the specificity of a bacterial strain [4].

More recently, a study was done utilizing a PDMS based microfluidic immunosensor chip integrated with nanoporous alumina membranes to detect *E. coli* and *Staphylococcus aureus* food pathogens [9]. The idea was to measure the impedence amplitude of the nanopores once the bacteria attached to their respective antibodies immobilized on the nanoporous alumina membranes. These membranes have been adapted from DNA hybridization detection methods and aimed to increase the sensitivity of biosensing with smaller concentrations of bacteria. This method proved to be successful at a concentration as low as 10^2 colony-forming units (CFU)/mL. In lieu of this success, questions are still raised about the amount of bacteria that can actually bind to antibodies due to factors such as affinity between this particular antibody and bacteria antigen, random orientation of antibody molecules during immobilization process, and the antibody

grafting efficiency on the silane layer [9].

With the analysis of these studies, there have been many that prove to have potential of reaching the goals to improve efficiency and lessen time necessary to carry out successful experiments. However, few seem to address important concerns about the detection of septicemia. Even though sensitivity has improved to about 10^2 cells/mL, no studies have shown the incorporation of laser technology to detect septicemia. Furthermore, the detection capabilities generally present to be underdeveloped for this study's purposes and simply require more testing. Studies that boast sensitivity lack the specificity identification of even primitive PCR assays. In a long-term view, the implementation of an efficient bacterial detection system for septicemia screening must have the versatility to efficiently detect more than one type of bacteria. Since there are so many causes of the symptoms that present with septicemia, the system must also be able to determine if the cells detected are at all viable cells that are actually responsible for the deteriorating health of a patient. With the development of this study, there holds the potential to address these critical issues. A closer look into the microfluidic system used in this study reveals the advantages of utilizing photoacoustic detection of *E. coli*.

Photoacoustic Detection

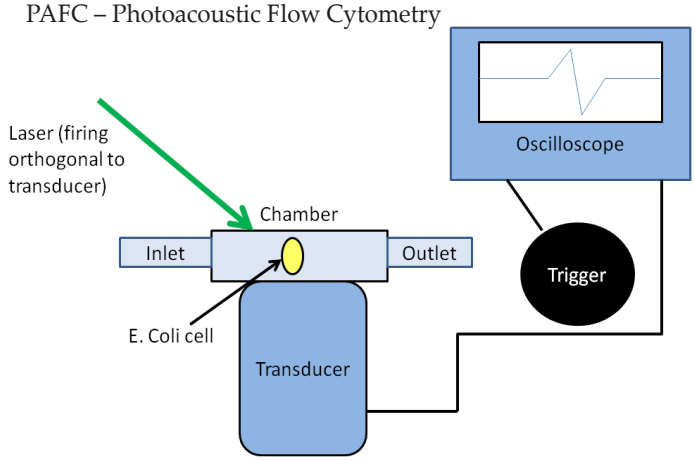
The photoacoustic flowmeter developed by the lab of Dr. John A. Viator, adapted from the detection of circulating melanoma cells, allows us to detect *E. coli* cells. The system uses a rapid pulsing wavelength tunable laser that fires onto the absorbing particles. The absorption of light causes the particles to undergo thermoelastic expansion, resulting in pressure waves that are then detected by an acoustic sensor. We collect the samples and image them to confirm the *E. coli* detection [15].

METHODS

Abbreviations

PA – Photoacoustic

PAFC – Photoacoustic Flow Cytometry



Schematic of Photoacoustic Detection System

Photoacoustic Detection System

As shown above, the photoacoustic detection system starts with the addition of a sample in the inlet that passes through the chamber and is irradiated by the laser light firing orthogonal to the transducer. The transducer picks up the signal as the sample passes through the chamber. The trigger detects the presence of a pressure wave that then shows up as a waveform on the oscilloscope. The sample then leaves through the outlet section of the chamber [15].

Research Design and Methods

This experimental design explores tagging *E. coli* cells with dyed microspheres and gold nanoparticles. Once a method is developed to label the *E. coli* cell, PA contrast agents are attached to the cell and the characteristic PA signal response from the cells is measured. Moreover, the PAFC system is optimized to detect cells of artificially pigmented *E. coli*. *E. coli* is also added to and subsequently separated from healthy blood samples. These results will be applied to test a model for the detection of infectious pathogens in low concentrations in human blood to suggest a new diagnostic tool for sepsis.

In order to develop a consistent labeling protocol using immunochemistry of the *E. coli* cell, surface antigens must be identified and targeted with antibodies attached to fluorescent particles. Since this procedure is being developed in order to be applied to PA contrast agents, the fluorescent particles chosen to be a model for the contrast agents must be similar in size (30-300nm) and the chemistry must be easily transferrable to the contrast agents as well. Both confocal and fluorescent microscopy is used to quantify specificity, level of attachment, and cellular localization in order to understand the distribution of particles on cell surface and validate the technique of attachment. The use of fluorescent particles in this experiment is for the purpose of verifying the results and would not necessarily be part of the clinically implemented photoacoustic technique.

Characterization of the PA signal from just the contrast agent as well as the cell bound contrast agents helps validate the feasibility of the hypothesis. The proposed PA contrast agents are gold nanoparticles (AuNPs) and dyed polystyrene nanobeads. AuNP nano-rods are the best choice due to their dual peak absorption in the optical wavelengths. This interesting characteristic is used to distinguish these nano-rods optically, and therefore photoacoustically, from other materials. Additionally, dyed polystyrene beads can be found in many different colors which define specific absorption spectrums of the different particles. After the characterization of the free and cell bound contrast agents, the detection limit and sensitivity of photoacoustic flow cytometer to the artificially pigmented *E. coli* cells will have to be measured.

Healthy vials of blood in 5mL samples will be taken in order to spike and then separate the E.coli and white blood cell layer from the whole blood. Histopaque 1077, used to separate the white blood cell layer from the red blood cell layer, is added to 15 mL Falcon tubes and a sample of blood spiked with *E. coli* at concentrations from 10^5 to 10^3 of bacteria cells will be added on top of the Histopaque.

A. Conjugation Procedure:

Since AuNPs and dyed polystyrene beads of 30-300nm are used as the photoacoustic contrast agents, a red fluorescent polystyrene nanobead of 300nm are used to develop the conjugation procedure. Like the contrast agents, the blue microsphere and the AuNPs are functionalized with

streptavidin groups in order to carry out biotin-streptavidin conjugation procedure to attach the antibody protein to the fluorescent particle. The success of the procedure is validated with the fluorescent imaging. The *E. coli* cell surface is targeted with anti-*E. coli* antibodies which are conjugated with each particle. Additionally, biotinlyated antibodies and streptavidinated nanoparticles were purchased to ensure that the conjugation procedure is successful.

B. Experimental Design:

There are three separate control groups to be differentiated from each other, as well as, from the experimental group. Control Group 1 is *E. coli* incubated with nothing and modified in no way. Control Group 2 is *E. coli* cells incubated with just fluorescent particles which are not attached to antibodies; this tests if the particles by themselves have any natural affinity for the bacteria cells. Control Group 3 is *E. coli* cells with anti-*E. coli* surface antigens blocked and then incubated with fluorescent particles conjugated with either anti-*E. coli*; this will verify that only targeted cell surface receptors are affected by the procedure. The Experimental Group is *E. coli* cells incubated with fluorescent particles conjugated with the antibody; this validates that the particles attached as expected.

C. Fluorescent Imaging:

A confocal fluorescence capable microscope is used to visualize the general attachment of particles to the cells in all groups. The experimental group should show robust fluorescence while the other control groups should show little to none. Confocal microscope is used to find the localizations of the particle on the cell in order to see if the large number of particles are on the cell membrane or have been internalized by the cell. The particles are expected to localize on the cell membrane but internalization could affect results.

D. Quantification:

Average fluorescent intensities from multiple cells are measured and compared with stock solution of fluorescent particles in order to calculate possible number of particles on the cell surface. This can be used in the future to estimate the expected PA contrast agent concentrations on the cell surface.

E. Photoacoustic Response from Contrast Agents:

The photoacoustic spectrum in the optical wavelengths of free AuNP rods with peak absorptions at 530nm and 584nm will be obtained by irradiating each sample at 532nm. The same will be carried out for red fluorescent blue microspheres. This wavelength is used because it is generally acceptable standard for PA detection, particularly with two different color particles. This will help characterize the contrast agents and their respective PA responses.

F. Attachment and Characterization of Contrast Agents on Cell Membrane:

The AuNPs and polystyrene nanobeads are functionalized with streptavidin groups similar to the fluorescent particle used to develop the labeling procedure. A slightly modified, if not the exact same, chemistry is used to attach the contrast agent with the antibody of choice. This then is incubated with the cell according to the procedures previously established in the labeling procedure. The control group is receptor blocked E. coli cells which are incubated with the contrast agents which target anti-E. coli. The experimental group are E. coli cells incubated with the antibody bound contrast agents. Both groups are cleaned of free floating contrast agents after incubation time. Little to no PA signals is expected from the control groups while robust PA responses are expected from contrast agent targeted cells.

G. Sensitivity and Detection Limit of Photoacoustic Flow Cytometry:

A previously developed PAFC system is used with the artificially pigmented E. coli cell to determine the detection limit and sensitivity. We need to be able to detect tagged E. coli cells at concentrations as low as 10^2 cells/mL based on the detection limits of other comparable procedures.

H. Data Analysis:

In order to analyze the waveforms collected, we calculate the rectified integrals of each of the pressure waves. These calculations measure the absolute value of the area under the curves to confirm which sample gave the highest results, and therefore the best signal. The SNR (signal to noise ratio) is also measured to use in conjunction with the rectified integrals. This measures how different the signal was from the inevitable noise emitted by the system.

RESULTS

Cell Tagging

The Blue Microspheres and the Gold Nanoparticles both were able to tag to the E. coli cells through the conjugation process using the streptavidin and biotin bond of the nanoparticles, the anti-E. coli antibodies, and the E. coli cells. This process is schematically shown in Figure 1.

Imaging

The cell conjugation was further confirmed by the imaging of the red fluorescent BMS on the E. coli cells with the confocal microscope. An image of the tagged E. coli at 60X magnification is shown in Figure 2.

Photoacoustic Detection

After running a sample of PBS for a baseline, a sample of E. coli tagged with only BMS, and a sample of *E. coli* double tagged with both BMS and AuNP, we collected 20 waveforms from each data set. The middle sets of data for each sample are represented in Figures 3.1-3.3. The measurements from the integrated pressure waves and SNRs are shown in Figure 4 and Table 1.

DISCUSSION

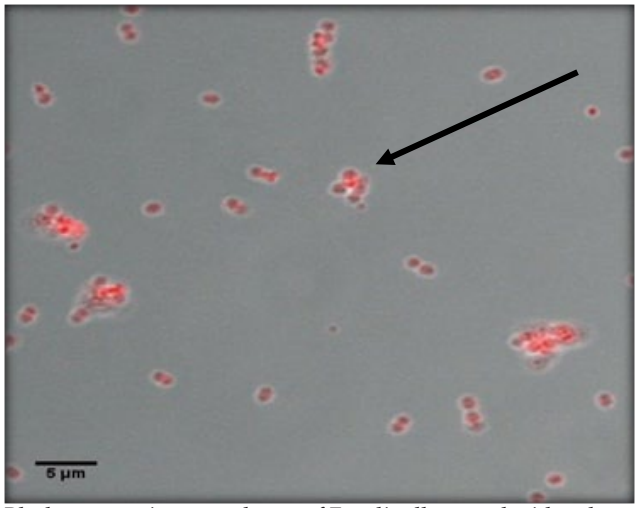
It appears from the results that the double tagged and the BMS tagged *E. coli* sample responses were both well above that of the control. It is also suggested that the BMS sample had the greatest photoacoustic response; greater than the PBS baseline

and even the double tagged sample with BMS and AuNP. Against what would typically be expected, these results imply that it is more beneficial to use only the BMS conjugate with the *E. coli* cells instead of both BMS and AuNPs. Both the rectified integrals and the SNRs further confirm the suggestion that the best signals were created by the *E. coli* tagged only with the BMS. The reason for the double tagged sample of *E. coli* was because the use of both BMS and AuNP increases the workable surface area of the *E. coli* cells and allows more things to attach to the cells, ultimately creating the opportunity for a stronger signal. We believe that the main contributing factor to these unexpected results was the way in which we used the photoacoustic detection system. Typically, as illustrated in Figure 5 below, the system is used with two-phase flow, pumping sample and air successively through the chamber. This steady flow kept the chamber clear of cells that may want to stick to the inner surface of the chamber, eliminating any constant spikes picked up on the oscilloscope. However, the two-phase flow aspect was not needed for this experiment. Additionally, our system is under patent and we are in the process of making the chambers that we use more permanent. We believe that the constant flow of material—though the chamber was cleaned in between each sample—created a charge that allowed cells to stick to the inner surface and leave a constant spike that showed up in the results of the BMS test, since it was run last. With the creation of chambers that cater to our specific needs, and perhaps, individual chambers for each test run, we will be able to secure more accurate results.

LIMITATIONS AND FUTURE WORK

Some of the limitations involved with this project are that we were only able to detect *E. coli* at a concentration of about 10 million cells/mL. In order to make sure that we are able to accurately determine whether or not a person has *E. coli* in their blood, we have to be able to detect lower concentrations of cells. The numbers of viable cells in blood vary and symptoms can be seen even with a concentration as low as 1 cell/10 mL. Also, we were only able to run the test once because of equipment and time restraints. Once we have improved our methodology we will be able to run several additional tests that will either confirm or refute the results obtained. After better results are collected that show the actual detection of the *E. coli* samples, we will be able to begin the process of spiking healthy blood samples and determining a good separation process so that we can isolate the layer of blood where the *E. coli* cells will present. This process will be beneficial in attempting to mimic the conditions of the use of actual patient blood.

Figure 2



Black arrow points to a cluster of E. coli cells tagged with red-fluorescent BMS.

Figure 1

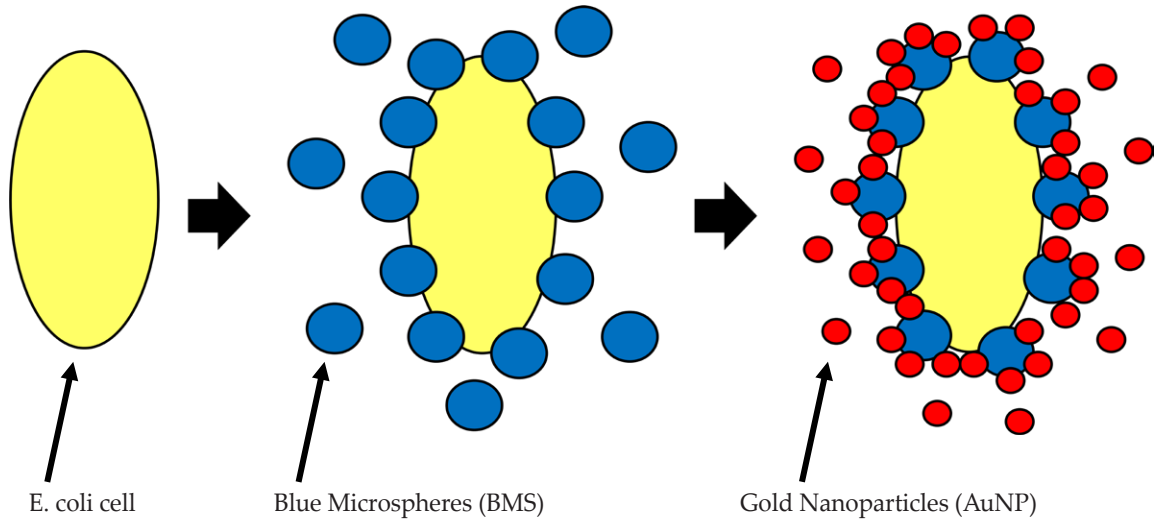


Figure displays a schematic of an E. coli cell and the conjugation process with BMS and AuNPs.

Figure 3.1

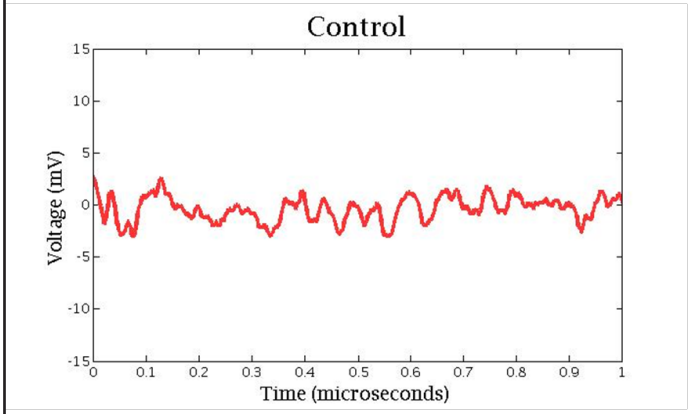


Figure 3.2

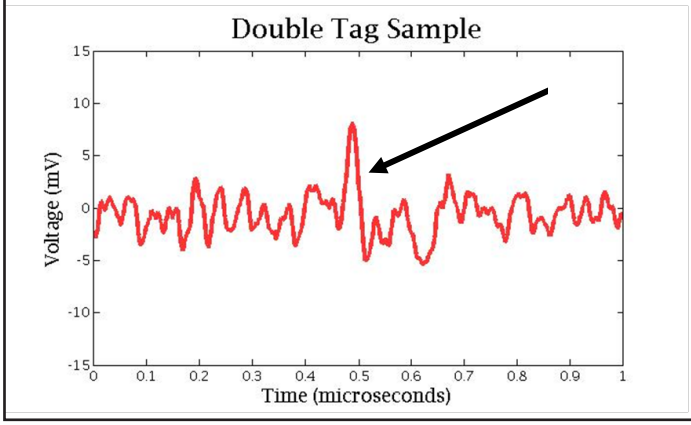
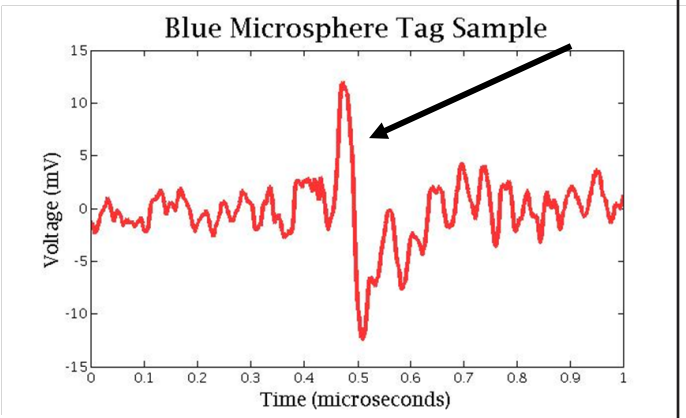


Figure 3.3



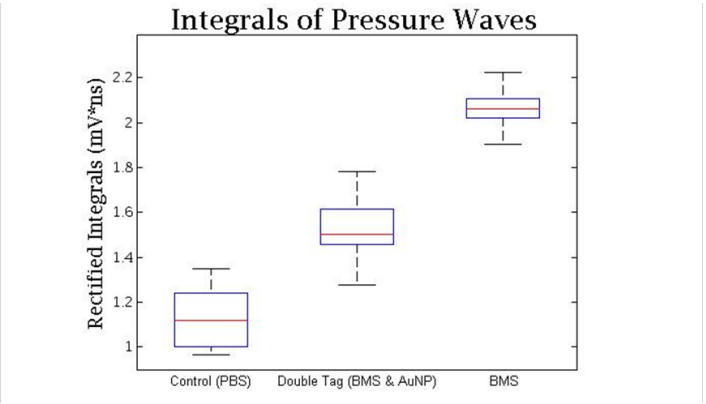
The black arrows point to the Photoacoustic event displayed as a waveform on an oscilloscope.

Table 1

Signal to Noise Ratio			
	Control	Double Tag	BMS
Avg SNR	1.15 : 1	2.16 : 1	3.39 : 1

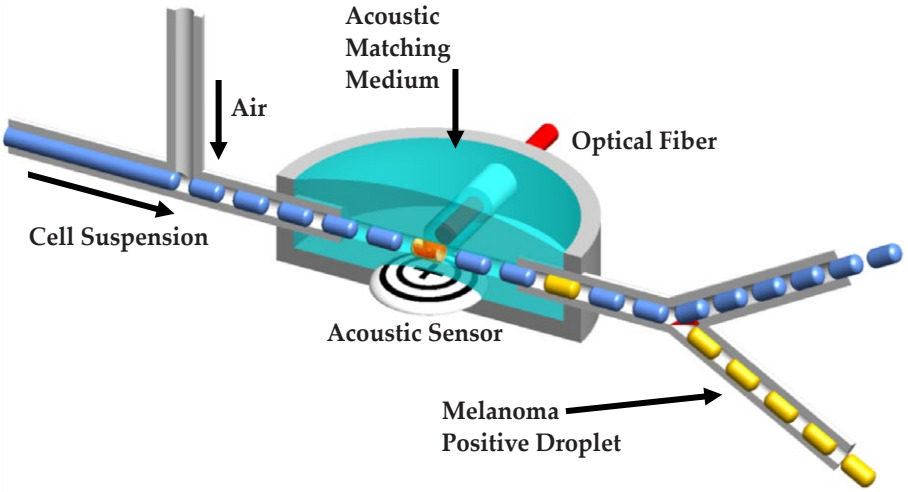
Table displays the average SNRs for each of the data sets.

Figure 4



Graph shows a box plot representation of the integrals of the pressure waves of each data set.

Figure 5



Schematic of the Two-Phase Flow aspect of the Photoacoustic Detection System used for melanoma detection

ACKNOWLEDGEMENTS

Special thanks to: Kiran Bhattacharyya, Benjamin Goldschmidt, Kyle D. Rood, other Viator Lab members, Dr. Shramik Sengupta, Dr. Heather Hunt, the MU Office of Undergraduate Research, and the McNair Scholars Program.

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Brand Loyalty: A Matter of the Maximizing Trait and Product Category Involvement?

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ABSTRACT

For many companies, a small but very loyal part of their overall consumer base is responsible for the majority of their profits, so the concept of brand loyalty is of considerable importance. The preponderance of research on this subject has focused on identifying the role of marketer-controlled factors in fostering brand loyalty. However, it is also important to consider the *consumer*. This study investigated the relationship between two individual-difference traits of consumers, the maximizing trait and product category involvement, and brand loyalty. 250 undergraduate students participated in an online survey that measured the aforementioned constructs. Brand loyalty and product category involvement were measured over three product categories -- consumables, represented by toothpaste, semi-durables, represented by athletic shoes, and technology, represented by mobile telephones. For the maximizing trait, the standard-setting component was measured with a three-item scale. Results showed a statistically significant positive relationship between brand loyalty and product category involvement in all three product categories and a trend in the data for a positive association between brand loyalty and the maximizing trait.

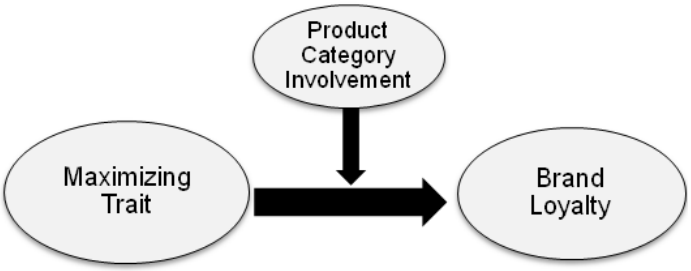
INTRODUCTION

For many companies, a small but very loyal part of their overall consumer base is responsible for the majority of their profits, so the concept of brand loyalty is of major importance. The preponderance of research on this subject has focused on identifying marketer controlled factors. However, it is also important to consider the *consumer*. The present study explores the relationship between brand loyalty and two individual-difference variables, the maximizing trait and product category involvement.

One of the traits in this study, maximization, is relatively new. This concept states that whenever an individual is faced with a decision he or she will act either act as a maximizer or a satisficer. A maximizer is someone who is always striving to make the best possible decision, whereas a satisficer is someone who is happy with a "good enough" option as long as it meets his or her minimum standards (Chowdhury et al., 2008). The other individual-difference variable that I investigate as a part of this study is product category involvement. A consumer who finds a product or activity that is important to or relevant to his or her values, needs, or interests is considered to have a relatively high level of involvement with it (Peter, 2010; Zaichkowsky, 1985). This study examines relationships between these two individual-difference variables

and brand loyalty (see Figure 1), in three product categories - consumables, represented by toothpaste, semi-durables, represented by athletic shoes, and technology, represented by mobile telephones. The issues addressed in this study have considerable significance for companies seeking to build long-term relationships with their consumers.

Figure 1
Conceptual model for the effects of two individual-difference variables on brand loyalty



CONCEPTUAL BACKGROUND

Brand Loyalty

In a marketplace categorized by an abundance of choice, firms are faced with the daunting task of finding a way to set their products apart from the rest. Brand loyalty is a continuing point of interest because loyal, repeat-purchase consumers result in positive market outcomes for firms, including larger market share, profits, and return on investment (Tam et al., 2009). Loyalty is also important because customers are a lot less costly to retain than to attract (Aaker, 1991). Throughout past research brand loyalty has been defined a number of ways but for the purposes of my study, I will adopt one of the more common definitions surrounding brand loyalty, developed by Oliver (1997), "a deeply held commitment to re-buy or re-patronize a preferred product or service consistently in the future, despite situational influences and marketing efforts having the potential to cause switching behavior". This definition outlines the two major aspects necessary for understanding brand loyalty: the *dimensions* of brand loyalty and brand *attitude strength*.

The first aspect of the concept of brand loyalty is its two dimensions – behavioral and attitudinal. Behavioral (or purchase) loyalty focuses on the actual repeat purchases a consumer makes. Conversely, attitudinal brand loyalty is concerned with a consumer's preference, intent, commitment, and disposition towards a brand (Chaudhuri, 2001). Although few studies have combined both of these dimensions (Russell-Bennett et al., 2007), I believe that understanding the interaction between both components of brand loyalty and other variables is vital to understanding this complex phenomenon. Many commonly used methods for measuring brand loyalty are one-dimensional and only capture the behavioral aspect of brand loyalty (Jacoby et al., 1973). These highly quantitative measures concern themselves only with a consumers' observable purchasing behavior and lead to the oversimplification of brand loyalty as simple repeat purchasing. While these types of behavioral measurements are undoubtedly useful, brand loyalty cannot be defined and measured singularly by the occurrence of repeat patronage or purchases.

The second major aspect of brand loyalty is relative attitude strength (Jensen et al., 2006), which becomes extremely important when a firm is attempting to predict consumer behavior in varying market conditions. The most important part of this component is attitudinal differentiation, which is the ability for a consumer to differentiate and find important differences among their alternatives. The second part of attitude strength is established in the last part of our definition of brand loyalty, "despite situational influences and marketing efforts having the potential to cause switching behavior" (Oliver, 1997). Stronger brand loyalties result in reduced variety- seeking tendencies and brand trial and increased resistance to other brands despite out of stocks, competitor promotions, and other situations with the potential to sway a consumer's intended purchasing decision (Jensen et al., 2006).

Understanding the motives behind purchases is critical to the ability of firms to create and deliver effective marketing strategies. For this reason, research around brand loyalty has tended towards areas that are under the control of or that can be potentially influenced by firms and marketers. However, my research will focus on two consumer individual difference variables, the maximizing trait and product category involvement, which are outside of a firm's control. Although these factors are uncontrollable for firms, understanding these factors can lead to a deeper understanding of consumers in general and provide insights into managing a relationship with them.

The Maximizing Trait

In his book "The Paradox of Choice", Barry Schwartz (2004) introduces us to the concept of the maximizing trait. When making decisions, a person tends to behave as either a maximizer or a satisficer. When a maximizer is faced with a decision, he or she performs an extensive search in order to find and select the best possible option. They very literally seek to "maximize" every decision they make. In contrast, when a satisfier is faced with a decision, he or she sets standards and parameters for what is acceptable. Once an option that meets the person's standards is found, a satisficer will end his or her search process without further thought as to the possibility of even better options.

Further research by Schwartz et al (2002) has found significant differences between maximizers' and satisficers'. There are positive correlations between maximization and regret, perfectionism, and depression and there are negative correlations between maximization and happiness, optimism, satisfaction with life, and self-esteem. Another difference is in the response to the increasing level of choice that consumers are faced with in almost every aspect of their lives. The added options have a negative effect on maximizers as they attempt to continue exploring all available options, but it has a neutral effect on satisficers.

Schwartz (2004) has also proposed a thirteen-item scale to measure the maximization trait in his book. However, it was later determined (Nenkov et al., 2008) that this scale actually measures three distinct components of the trait: alternative

search, decision difficulty, and high standards. Alternative search refers to the tendency of a maximizing individual to desire to keep looking for better options. Decision difficulty measures how difficult it is for an individual to choose among the available options. Finally, there is the high standards scale, which measures people's tendencies to hold themselves, and things in general, to high standards. The three items used to measure high standards are best fitting for the purposes of the present study.

Since the release of Schwartz's book, the maximizing trait has caught the attention of many behavioral scientists and many follow-up studies have been done. One of those follow-up studies examined the effects of the maximizing trait on a decision made under time pressure (Chowdhury, 2009). The study involved an experiment in which participants were asked to make gift purchase decisions under a time constraint. The maximizers perceived more time pressure than satisficers, regardless of whether they were given the small or large assortment. The researchers also found that maximizers were more likely to change their original time-constrained decision if given the opportunity to shop again without the time restriction. This study's results echo previous findings that maximizers are plagued by higher levels of decision regret and experience higher levels of stress from everyday choices.

Another article (Legouxet et al., 2010), on the behavioral differences of maximizers and satisficers leads me to consider the effect this trait may have on brand loyalty. The study defines the Sisyphus effect for maximizers as the tendency to downplay past experiences when making relevant future decisions. Maximizers were more likely to attempt to start completely over when making a purchasing decision by re-evaluating all of the available options. In a follow-up study, the researchers found that while maximizers experience more regret, it doesn't make as big of an impact on a maximizer's future decision considerations as it would for a satisficer.

In summary, previous studies have found substantial differences in the purchase decision process of maximizers versus satisficers. This study will seek to shed further light on the behavioral differences between maximizers and satisficers and their tendency to be brand loyal or not. There has not been a study on the maximizing trait and its possible effects on a profit-driving behavior like brand loyalty. In seeking to find a relationship between this trait and brand loyalty, I hope to demonstrate the managerial importance of the concept of the maximizing trait.

Product Category Involvement

For the purposes of this study, I will adopt a fairly influential definition from Zaichowsky (1985) which states that product category involvement, sometimes referred to as enduring involvement, is the perceived relevance of a product category to an individual. This relevance is often based on a person's values, needs, and interests and usually endures over an extended period of time. It is the general or base level of concern a consumer has for any item, event, or activity, not the specific evaluation a product is given. Involvement is important to firms because of the potential resulting behaviors that create important points of segmentation and differentiation for marketing strategies (Reimann and Aron, 2009). Highly involved consumers are more motivated to search for and study information in detail. Less involved consumers are more likely to rely on heuristics, simple decision rules to simplify the decision- making process.

Four different types of involvement have been defined in previous research and literature: enduring, situational, cognitive, and affective (Bloch et al., 1983; Peter, 2010). I will discuss the two types pertaining to this study - enduring and situational. The previous definition from Zaichowsky (1985) speaks to enduring involvement, which will be the focus of this study. Enduring or product category involvement refers to an ongoing interest that continues over a long period of time despite situational factors. It is caused by an inherent interest, value, or need that moves an individual towards an object or activity. This type of involvement usually occurs for only a select few items and can develop in numerous ways, like youth fascination or family heir.

Situational involvement refers to a temporary increase of a consumers' relevance or interest in a certain product category (Bloch et al., 1983; Peter, 2010; Zaichowsky, 1985). It is completely determined by the immediate environment and occurs during a specific time frame with an expressed start and end. This increase of involvement is usually the result of a high level of perceived risk. An example of a high situational involvement purchase is the purchase of a household appliance, which is not commonly an area of enduring involvement but typically causes high situational involvement because of the relatively high level of time and financial risk associated with the purchase. Situational involvement can also be increased as a result of sales promotions and discounts.

Involvement has been linked to brand loyalty in previous research (Suh et al., 2006). When involvement with a particular product category is high, there is a high level of developed attitudes towards advertisements and corporate images. This in turn leads to a high level of attitudinal brand loyalty for the brand that is perceived as the best. In contrast, in low involvement situations brand loyalty is rewarded to the brand that provided previous satisfaction. The researchers recommend that firms in typically low involvement categories focus on product quality, whereas firms in high involvement categories should invest in brand and corporate advertising.

In summary, previous studies have focused primarily on defining and measuring involvement, with more recent studies attempting to relate it to different market outcomes and consumer behaviors. This study will build on previous findings that indicate that high enduring involvement relates to high attitudinal brand loyalty and look at the interaction between involvement level and the maximizing trait, and the effect of that interaction on brand loyalty.

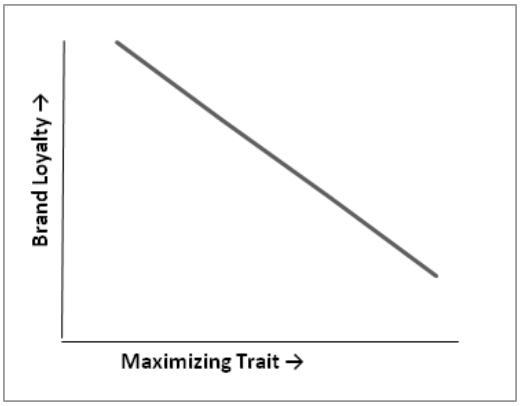
HYPOTHESES

Maximizers are intent on always making the best possible decision or selecting the best option in any purchasing situation, and engage in extensive searches in order to do so (Schwartz, 2004). The aspect of maximizers most likely to affect brand loyalty is the Sisyphus effect (Legoux, 2010). The Sisyphus effect is the fact that when maximizers are faced with a purchasing situation, they are less likely to consider a relevant previous purchase outcome, be it positive or negative. This means that maximizers, relative to satisficers, are more likely to start completely over

when making a purchasing decision and re-evaluate all of the available alternatives for the purchase. So even if a maximizer has a positive experience with a brand, that will not sway future decisions. Satisficers on the other hand, will consider previous satisfaction or dissatisfaction when making a purchase and will allow that evaluation to make an impact on future purchase decisions. So if a satisficer has a positive experience with a certain brand, he or she will use that information in future decisions. Given this discussion, it is expected that the mindset of a maximizer will inhibit his or her ability and desire to remain loyal to a particular brand and will result in a negative relationship between the maximizing trait and brand loyalty (See Figure 2).

H_1: There will be a negative relationship between brand loyalty and the maximizing trait.

Figure 2
Hypothesis regarding Maximizing Trait and Brand Loyalty

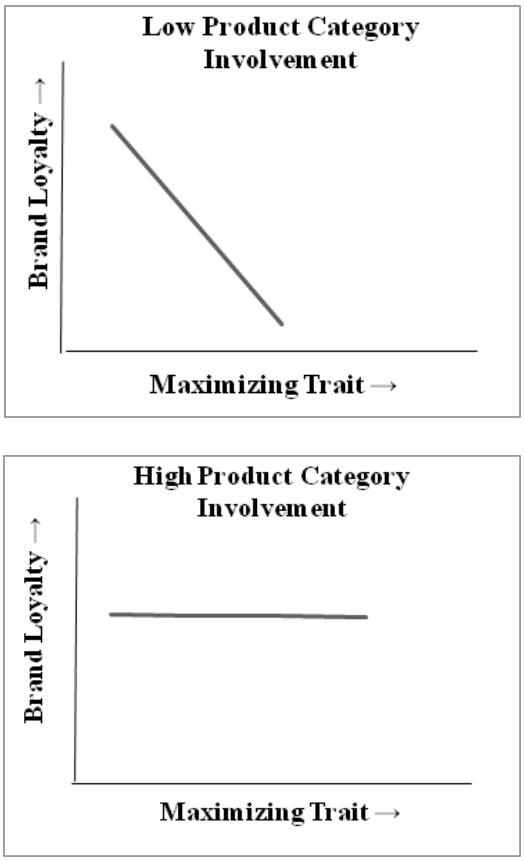


Next, I consider the interaction of the maximization trait and product category involvement. When involvement is low, there are few if any developed attitudes surrounding a brand and any loyalty to a brand is based solely on previous satisfaction with a brand. It is therefore expected that the previously discussed effects of the maximizing trait will influence the decision similarly to the manner discussed in H_1. If the individual is a maximizer there will be a low level of brand loyalty. The maximizer will not consider previous satisfaction in his or her decision and therefore there will be a lower level of brand loyalty. A satisficer on the other hand will consider previous satisfaction and will have a higher level of brand loyalty (See Figure 3).

When product category involvement is high, there are already developed attitudes surrounding the brand and corporate image that will influence brand loyalty (Suh et al., 2006). In this situation, the attitudes that have been developed as a result of the high level of involvement will have greater influence on the purchasing decision than the individual's maximizing and satisficing tendencies. Previous research has found a positive relationship between brand loyalty and product category involvement. So in a high involvement situation, brand loyalty is expected to be uniformly high regardless of whether the person is a maximizer or a satisficer (See Figure 3).

H_2: When product category involvement is low, there will be a strong negative relationship between brand loyalty and the maximizing trait. However, when product category involvement is high, there will be a weak or no relationship between the maximizing trait and brand loyalty.

Figure 3
Hypotheses regarding joint effects of the maximizing trait and involvement on brand loyalty



METHOD

Overview

To gather the data needed to test my hypotheses, an online survey using Qualtrics survey software was created and administered. 250 University of Missouri undergraduate students completed the survey for extra class credit. Three previously developed and validated scales were utilized to measure the three constructs of interest in my hypotheses – the maximization trait, product category involvement, and brand loyalty. Brand loyalty and product category involvement were measured over three product categories – consumables, represented by toothpaste, semi-durables, represented by athletic shoes, and technology, represented by mobile telephones. After the measures for these three constructs, socially desirable responding was also measured in an attempt to identify respondents who were not completely truthful in their responses. Lastly, demographic information was collected. In order to minimize demand effects, which occur when a respondent is able to guess the purpose of the research and changes his or her responses as a result, the survey

participants responded to the measures for brand loyalty, the dependent variable, first, then the measures for the maximizing trait, product category involvement, and socially desirable responding, respectively. Sets of filler questions were inserted between the measures for each of the constructs in order to avoid carry-over effects from the measures of one construct to another.

Procedure

Research participants signed up on a website to take part in the survey for extra credit. They participated in groups of 12-20. When participants arrived at the lab, they were instructed to sit at one the provided laptops with wireless internet connection. They were provided a letter with instructions for the survey and the URL where the survey was available. After reading the letter, participants typed the URL into the laptop's internet browser, completed the self-paced survey, and were allowed to excuse themselves whenever finished.

Measures

Details of the measures, including a complete list of scale items, can be found in the Appendix. Brand loyalty was measured with four Likert-scale (agree-disagree) items for each of the aforementioned product categories (Chaudhuri et al., 2001; Feick et al., 2003). Before each set of brand loyalty questions, participants were asked a set of questions to determine if they were consumers in the product category of interest and also if they were responsible for purchase decisions in the product category. If the respondents were consumers *and* responsible for the purchase decisions, they were then asked to enter the brand name of the product they were currently using in the category of interest. That brand name was then automatically inserted by the survey software into each of the four brand loyalty items in the scale for that product category. This procedure ensured that the brand name was salient to the participants as they were responding to the brand loyalty measure. Next, after responding to a set of filler questions, respondents were given the three Likert-scale (agree-disagree) items in the standard-setting-subset of Schwartz's maximization scale (Nenkov et al., 2008; Schwartz, 2004). After that, respondents again were given a set of filler questions before responding to three Likert- scale (agree-disagree) items for product category involvement. This was measured only over the product categories in which the participants were consumers and the purchase decision makers (Stokburger-Sauer et al., 2012). Finally, socially desirable responding was measured using five (very true- not all true) items (Paulhus, 1988).

RESULTS

Measures for brand loyalty, product category involvement, and the maximizing trait were created by taking the averages of the items in their respective scales. Before beginning the analysis, participants' scores for socially desirable responding were compared to the sample average and two outliers on the high end of the scale were removed. Five respondents with extremely low survey durations were removed as well since it appeared they had not put sufficient thought into the task. Descriptive statistics such as mean, standard deviation, Cronbach's alpha, and measure correlations for all three product categories are shown in Tables 1.1- 1.3. The alpha values for all three scales were above the acceptable .7 level, which indicates that the scales are reliable.

Table1.1, which has the descriptive statistics for toothpaste, shows a marginally significant positive correlation between brand loyalty and the maximizing trait and a highly significant positive correlation between brand loyalty and product category involvement. Table 1.2 for athletic shoes shows a highly significant positive correlation between brand loyalty and the maximizing trait as well as between brand loyalty and product category involvement. Finally, Table 1.3 for mobile phones shows that there is only a highly significant positive correlation between brand loyalty and product category involvement.

Table 1.1
Descriptive statistics and correlations between measures for toothpaste category

Variable	**Mean**	**SD**	**Alpha**	**1**	**2**
1.Brand Loyalty	4.88	1.29	.86		
2. Maximizing Trait	5.55	1.02	.73	.13*	
3. Product Category Involvement	3.43	1.38	.77	.33**	.12

Table 1.2
Descriptive statistics and correlations between measures for athletic shoes category

Variable	Mean	SD	Alpha	1	2
1.Brand Loyalty	5.28	1.36	.92		
2. Maximizing Trait	5.52	1.012	.73	.29**	
3. Product Category Involvement	4.29	1.52	.92	.39**	.10

Table 1.3
Descriptive statistics and correlations between measures for mobile phones category

Variable	Mean	SD	Alpha	1	2
1.Brand Loyalty	4.86	2.00	.96		
2. Maximizing Trait	5.52	1.02	.73	.13	
3. Product Category Involvement	5.48	1.26	.86	.39**	.13

Notes: 1.* indicates correlation is significant at the 0.05 level (2-tailed).
2. ** indicates correlation is significant at the 0.01 level (2-tailed).

After looking at the descriptive statistics, I tested my hypotheses with multiple regression models and t tests. Table 2 shows the regression results for all three product categories in this study. Regarding my hypothesis H_1 of a main-effect relationship between brand loyalty and the maximizing trait, the only statistically significant result is in the opposite direction

of my prediction. It is also important to point out that my hypothesis H_2, regarding an interaction between the maximizing trait and product category involvement is not supported by the regression results for any of the three product categories. The results show that the overall regression model for toothpaste was highly significant, $F(3,220) = 9.87$, $p < .001$. It also shows that there is a statistically significant positive coefficient for involvement. All of the other coefficients are positive but not significant. Similarly for athletic shoes, the overall model is highly significant, $F(3,214) = 19.46$, $p < .001$. For this product category the coefficients for the maximizing trait and product category involvement are positive and significant. Finally for mobile phones, the model is again highly significant overall, $F(3, 196) = 13.21$, $p < .001$. The coefficient for product category involvement is the only one that is positive and significant. All other coefficients are positive but not statistically significant.

Table 2
Regression Results for Brand Loyalty

Independent Variable	BL-Toothpaste (N= 224)	BL-Athletic Shoes (N= 218)	BL-Mobile Phones (N=200)
Maximizing Trait	.13	.33**	.17
Product Category Involvement	.29**	.32**	.62**
Maximizing Trait X Product Category Involvement	.03	-.04	.04
Adjusted R2	.11**	.20**	.16**
F Statistic (for overall model)	9.87**	19.46**	13.21**

Notes: 1. ** indicates $p < .001$
2. Cell values show unstandardized regression coefficient values.
3. Mean –centered values were used for the independent variables in the regressions.

To explore the data further, I looked separately at high vs. low involvement groups and maximizers vs. satisficers. For product category involvement, I performed a median split and compared the mean brand loyalty scores for the high vs. low involvement groups. For the maximizing trait, I took the top and bottom thirds of the scores on this scale to represent maximizers vs. satisficers and compared the mean brand loyalty scores of the two groups. Figure 4.1 shows the results for toothpaste. There is a marginally significant difference in brand loyalty between satisficers and maximizers ($t(150) = 1.65$, $p < .10$; $M_{Sat} = 4.75$ vs. $M_{Max} = 5.08$). There also is a highly significant difference in brand loyalty between the low and high involvement groups ($t(225) = 4.76$, $p < .001$; $M_{LowPI} = 4.53$ vs. $M_{HighPI} = 5.31$). Figure 4.2 shows the results for athletic shoes. There is a highly significant difference in brand loyalty between both satisficers and maximizers ($t(151) = 3.51$, $p < .001$; $M_{Sat} = 4.87$ vs. $M_{Max} = 5.62$) and the low and high involvement groups ($t(225) = 6.46$, $p < .001$; $M_{LowPI} = 4.79$ vs. $M_{HighPI} = 5.86$). Lastly, figure 4.3 shows the results for mobile phones. There is a marginally significant difference in brand loyalty between satisficers and maximizers ($t(134) = 1.80$, $p < .10$; $M_{Max} = 4.52$ vs. $M_{Sat} = 5.13$) . However, there is a highly significant difference in brand loyalty between low and high involvement groups ($t(201) = 3.49$, $p < .001$; $M_{LowPI} = 4.48$ vs. $M_{HighPI} = 5.46$).

Figure 4.1
Brand Loyalty for Toothpaste Category

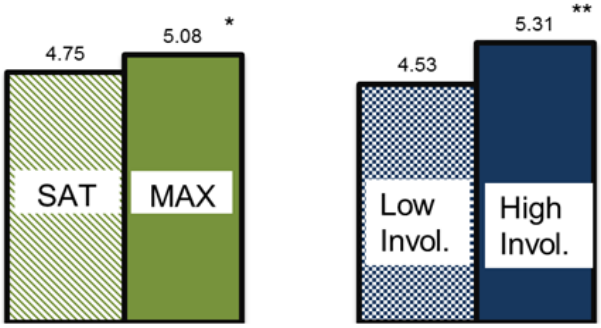


Figure 4.2
Brand Loyalty for Athletic Shoes Category

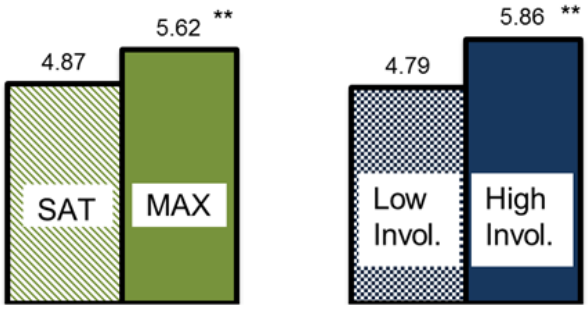
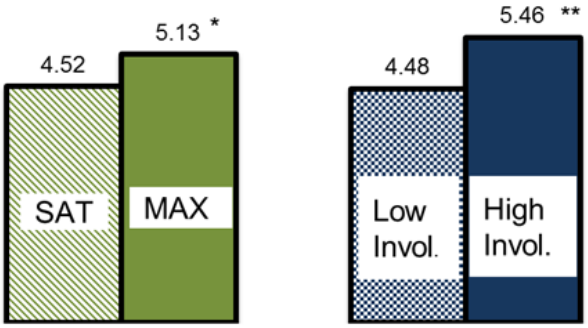


Figure 4.3
Brand Loyalty for Mobile Phones Category



Notes:
1. ** indicates $p < .001$; * indicates $p < .10$;
2. Brand loyalty was measured on a 1-7 point scale.
3. Comparisons for Satisficers vs. Maximizers are based on top and bottom third of distribution (N= 168).

DISCUSSION

The overall objective of this study was to explore the possible relationships between brand loyalty (Chaudhuri et al., 2001; Feick et al., 2003), the maximizing trait (Schwartz, 2004), and product category involvement (Peter, 2010; Zaichkowsky, 1985).

Although the majority of previous research on this subject has focused on identifying the role of marketer-controlled factors in fostering brand loyalty, it is also important to consider the *consumer* as a possible area of significance for companies seeking to build long-term relationships with their customers.

My empirical study yielded several interesting results. Consistent with the findings of previous research, I found a significant positive relationship between product category involvement and brand loyalty in all three product categories. This means that the more involved an individual is with a particular product category, the more likely that person will be loyal to a particular brand. For example, a consumer who is very interested in cars, is likely to be loyal to a particular brand like Ford. But contrary to my expectations, there was a trend in the data for a positive rather than negative association between brand loyalty and the maximizing trait. This means that the greater maximizing tendencies you have in your decision making, the more likely you are to remain loyal to a brand once you find one that you believe is the optimal choice. As previously mentioned, my hypotheses regarding the interaction between the maximizing trait and product category involvement were not supported in any of the product categories. The other result of importance is that the regressions showed that the maximizing trait and product category involvement each make independent contributions to brand loyalty. This is important because although the relationship found between the maximizing trait and brand loyalty is a weaker trend, the maximizing trait does appear to contribute to respondents' overall level of brand loyalty.

My data replicate previous research findings on the relationship between brand loyalty and product category involvement. I would speculate a possible reason for the positive relationship between the maximizing trait and brand loyalty is the amount of time and effort a maximizer puts into the decision-making process. When a maximizer is faced with a decision, he or she attempts to imagine all of the possibilities and systematically researches and rules options out before deciding on the optimal choice. After this process is complete, the maximizing consumer likely has in-depth of knowledge about the choice options in the category, similar to that of a high involvement consumer, and therefore has reason to believe that he or she has made the best possible choice and that choice would hold true in the future.

There are several marketer implications. This work advances the body of knowledge surrounding brand loyalty and indicates that individual-difference traits are important factors to consider during segmentation and targeting. In my study both maximization and high involvement had positive relationships with brand loyalty. Therefore, it would be profitable for marketers to focus on high involvement and maximizer consumers. This could be achieved through the offering of more opportunities to learn about and experience the brand. However, additional research is needed to identify ways to demographically profile consumers in order to successfully target different marketing strategies based on the differences in the needs of these groups.

LIMITATIONS AND FUTURE RESEARCH

There are several limitations to this study. Firstly, the population from which my respondents were drawn was limited. All respondents were undergraduate University of Missouri students enrolled in Marketing 3000. Therefore, it would be interesting to see if the results of this study are consistent when a more representative sample is surveyed. This study also used self-reported brand loyalty. Using some kind of behavioral measure of brand loyalty could also help validate the present findings. These results indicate the fact that the maximizing trait does play a role in shopping decisions, so it would be interesting to explore the possible relationship between the maximizing trait and other shopping variables, like internet shopping and the rise in opportunities for comparison shopping. Finally, a study that links these individual-difference traits with particular demographic segments that can be targeted by marketers would make these traits more relevant to mangers.

APPENDIX

Brand Loyalty (1-7, Anchored "Strongly Disagree" and "Strongly Agree")

1. I will stick with Brand X because I know it is best for me.
2. I am willing to pay a higher price for Brand X over other brands.
3. I will buy Brand X the next time I buy [Product Category].
4. I intend to keep purchasing Brand X.

Maximization Trait (1-7, Anchored "Strongly Disagree" and "Strongly Agree")

1. No matter what I do, I have the highest standards for myself.
2. I never settle for second best.
3. Whenever I'm faced with a choice, I try to imagine what all the other possibilities are, even ones that aren't present at the moment.

Product Category Involvement (1-7, Anchored "Strongly Disagree" and Strongly Agree")

1. I am very interested in anything related to [Product Category].
2. I value [Product Category] as an important part of my life.
3. [Product Category] means a lot to me.

Socially Desirable Responding (1-7, anchored "Not At All True" and "Very True")

1. I sometimes tell lies if I have to. *
2. I never cover up my mistakes.
3. When I hear people talking privately, I avoid listening.
4. I have said something bad about a friend behind his/her back. *
5. I don't gossip about other people.

Note: * indicates reverse-scaled items

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Development of a Sensor for the Detection of Explosive Compounds Utilizing Fluorescently Labeled Molecularly Imprinted Polymer Conjugates on Electrospun Scaffolds

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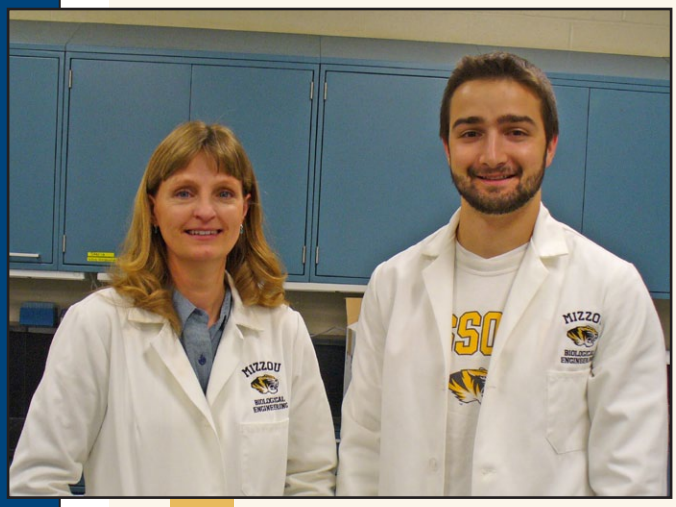

Damir Kolasinac originally hails from Vietz, Bosnia. As a senior in Biological Engineering, Damir was a recipient of the Lloyd E. Hightower Endowment Fund for Biological Engineering, member of the Tau Beta Pi Honors Society and Vice President of the Alpha Epsilon Biological Engineering Honors Society. This fall, Damir will pursue his master's in Mechanical & Aerospace Engineering at Oklahoma State University where he is conducting biomedical engineering research with blood contacting materials. His future career goals include working in research and development in the biomedical device industry.

ABSTRACT

The detection of improvised explosive devices is a vital concern for the department of defense and homeland security because they are responsible for over 50% of troop casualties in the Iraq and Afghanistan conflicts. With the development of an instrument that can detect their presence, the threats they pose may be dramatically reduced. The detection of nitroaromatic compounds, such as trinitrotoluene, has been highly researched through the molecularly imprinted polymer (MIP) technique and found to be highly effective when used as films. Electrospun scaffolds have been shown to create highly porous and large surface area membranes. This research investigates a novel approach of creating fluorescently labeled MIP conjugates on electrospun polymethyl methacrylate scaffolds as a means to detect aqueous 2,4-dinitrotolune. The sensors development and performance were tested utilizing FTIR, scanning electron microscopy, and fluorescence spectroscopy. The results revealed that MIP particles could be cross-linked onto PMMA scaffolds by utilizing sulfo-GMBS as a cross-linker. Additionally, the sensor functioned to detect the presence of DNT molecules by quenching the fluorescence of the sensing particles. A concentration of 0.07 mg/ml [MIP/ethanol] was determined to be the optimal concentration of sensor to scaffolds to create the largest limit of detection. The sensor's sensitivity could not be conclusively justified due to the heterogeneous distribution of sensor particles along the substrate and the limitations of our methodologies. This preliminary data expresses the feasibility of utilizing this approach to create chemical sensors.

INTRODUCTION

The threat from terrorist-made explosives is a high concern for the Department of Defense and Homeland Security. Improvised explosive devices (IEDs) pose some of the greatest dangers to troops and general public not only because of their destructiveness, but because of their intent to send a public statement and draw attention to the terrorist organization[1]. IEDs generally expel trace gasses containing nitroaromatic compounds and ample research is being performed to try to detect the highly explosive compound 2,4,6-trinitrotoluene (TNT)[2–5]. With the ability to detect TNT and locate IEDs, the potential threats they pose can be greatly reduced.

One technique that could be applied to detect TNT is to utilize molecularly imprinted polymers (MIPs), which produce molecular recognition sites [2–10]. The process involves the co-polymerization of a functional monomer, such as an acrylic, with a template, such as TNT, in the presence of a cross-linking agent,

such as ethylene glycol dimethacrylate. With the removal of the template molecule from the polymer matrix, specific binding sites are left behind that have precise recognition properties to the template molecule, and are ideal for chemical sensing applications. This property of molecularly imprinted polymers is highly advantageous over biological receptors due to their physiochemical stability and low cost [6].

Further advancements in molecular imprinting have led to the use of molecularly imprinted sol-gels as chemical and biological sensors, enzymatic and electro catalysis, immunoassays, and other applications [2], [3], [5], [10]. The use of sol-gels instead of polymer acrylics provides a highly versatile method. Sol-gel processing allows low temperature processing compared to conventional methods of glass manufacturing [2], [10]. The sol-gel process involves hydrolysis of silicon alkoxide precursors and catalytic polycondensation to produce a macromolecular network of siloxane bonds that surround template molecules [2], [10]. Following the removal of the template, highly sensitive recognition sites are left behind. The method can make recognition sites for virtually any analyte (i.e., sensing element) even if the natural receptor does not exist [4]. The molecular imprinting process of sol-gels is highlighted in Figure 1. The advantages of using the sol-gel method for the molecular imprinting of highly sensitive and selective matrices for analyte detection include: high purity, homogeneity, controlled porosity, stable temperature, and nanoscale structuring [2], [4].

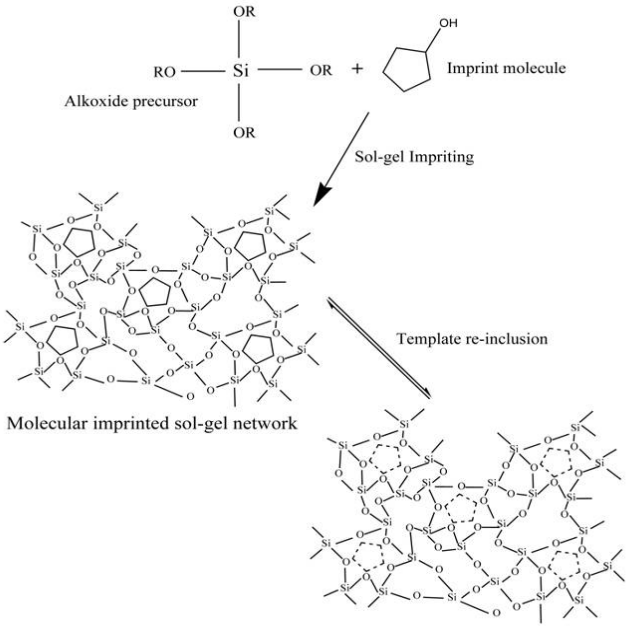

Figure 1: Description of molecularly imprinted sol-gel processing [2].

Sensors utilizing fluorescence based molecularly imprinted sol-gel have been found to be highly sensitive, upwards to the parts per billion (ppb) range [2]. Fluorescent dyes or fluorophores, such as fluorescein isothiocyanate(FITC) can be covalently linked to the sol-gel network due to the abundant binding sites in the sol-gel matrix. Nitroaromatic molecules act as electron acceptors, and thus can quench the emission of nearby excited fluorescence species upon binding to the molecular recognition sites [5]. These types of sensors are highly useful for the detection of TNT. While a myriad of TNT sensors have been developed by various research groups [3–5], the ideal TNT sensor remains elusive because criteria for the detection of minute traces of the compound, robustness to function in a variety of environments, low cost, and scalability for convenience of use [5] remain disproportionately unfulfilled.

Problems with MIPs include the heterogeneous distribution of the binding sites, poor site accessibility, and low capacity [6] that result in slowing of the kinetics of analyte binding [4] and leads to a loss in sensitivity. The importance of high surface-to-volume ratio at the nanostructures can result in better template removal, site accessibility, lower mass-transfer resistance, and well-defined materials' shape [4], therefore by utilizing a nanofabrication technique the problems associated with MIPs can be overcome.

One method to produce nanostructured platforms is electrospinning. Electrospinning is a highly popular process in which a high electric field is applied to the surface of a viscous polymer solution to induce a charge density on the surface. A force opposite of the surface tension is induced from mutual charge repulsion, which elongates the solution at the tip of the capillary tube and disperses the solution/fiber into a nonwoven mat on an electrically ground collecting surface [11]. The electrospinning technique is an extremely advantageous method for attaining membranes with a large surface area that are required to give higher flux and permselectivity through the membrane [7].

The development of a fluorescence based sol-gel sensors on an electrospun polymer matrix may be an improvement for TNT detection because it merges selective molecular recognition sites with nanostructured electrospun platforms. This work will focus on the development of such a sensor through the following approach: A fluorescently labeled nitroaromatic sol-gel solution will be evaporated to form a xerogel and then ground into a fine powder to increase the surface area of the sensing scheme. This powder will then be conjugated on to an electrospun poly(methyl methacrylate) (PMMA) nanofiber matrix that will provide a large surface area for the sensing molecules to attach and easy flux for analyte binding to occur. This work will continue the development of an ideal support ("i.e. one that has high surface area, interconnected pores, and open framework and that is mechanically and chemically stable" [10]) because breakthroughs in sensing systems have applications in a vast amount of fields due to the ease of creating polymeric recognition sites. Initially, DNT will be sensed due to its ease of preparation and safety.

MATERIALS AND METHODS

1. Materials:

The following chemicals were purchased: From Sigma-Aldrich (St. Louis, MO) methyltriethoxysilane, (tech., 90%), 3-aminopropyltriethoxysilane, 99%, acetonitrile, poly(methyl methacrylate) (PMMA)(M_W 120,000), fluorescein isothiocanate isomer (FITC), 1, 2,4-Dinitrotoluene, hexamethylene diamine, borate buffer tablets, acetic acid were purchased. 3-Mercaptopropyl trimethoxysilane was acquired from Fulka (United Kingdom). Hydrochloric acid and sodium hydroxide was obtained from Fischer Scientific (Fair Lawin, NJ). From

Thermo Scientific (Rockford, IL), the cross-linker N-[γ -maleimidubutyryloxy]sulfosuccinimide ester (sulfo-GMBS) was purchased. Acetone reagent ACS, 99.5% was acquired from Acros (NJ, USA). Other chemicals included 95% ethanol and 99.8% isopropanol from the University Chem Store.

A few of the chemicals were modified from their original composition. The borate buffer was titrated with hydrochloric acid (12.1 M) and sodium peroxide (10M) to lower the buffers pH to 8.0 and 11.5 respectively. Isopropanol and HCL were diluted to 95% and 1M respectively using ddH_2O.

2. Preparation of Molecularly Imprinted Polymer Sol-Gel Sensor:

A protocol for MIP TNT nanosensors was adapted from the paper by Holthoff et al. [3]. The protocol produces highly selective MIPs thin films for TNT. To create a larger surface area for analyte binding, the MIPs were crushed into fine particles using a mortar and pestle. The protocol has been subdivided in these categories:

Pre-hydrolysis of silane monomer: The following formulation was utilized to create the sol-gel mixture: 1.25 ml ethanol, 110 μl methyltrethoxysilane, 2.8 μl 3-mercaptopropyltrimethoxysilane, 3.5 μl aminopropyltriethoxysilane, and 6.25 μl 1M hydrocholoric acid were added to a beaker and stir vigorously with a magnetic stirrer for 30 minutes at room temperature. After stirring, the solution was transfer to a vial.

Addition of template and fluorescent dye: To the pre-hydrolyzed sol-gel mixture, 150 μl 10mM DNT in acetonitrile and 150 μl 10mM FITC in ethanol were added. This solution was vortexed for 30 seconds to achieve homogeneity and then the vial with the solution was wrapped in aluminum foil. (The fluoropore FITC is susceptible to photo-bleaching so it is vital to limit exposure to light sources during preparation and storage.) A small opening on top of the vial was left in order to allow the solution to evaporate slowly and solidify over a few days period. The times varied based on temperature and humidity. After a few days, the xerogel was scraped from the vial and crushed into a fine powder using a mortar and pestle.

Extraction of template: To remove the template, i.e., the DNT, from the xerogel, a batch of extraction buffer at a ratio of 8:2:1 (ethanol/acetonitrile/ acetic acid) was prepared. The crushed MIPs were immersed in the extraction buffer for 24 hours to remove the template.

3. Preparation of PMMA Solution for Electrospinning:

Electrospun PMMA fibers were utilized as the substrate for MIP immobilization. The preparation of the PMMA solution to be electrspun was adapted and modified from the dissertation of Craig Weilbaecher[12], where the electrospinning procedure for PMMA was optimized in order to obtain uniform and continuous fibers. One gram of PMMA was dissolved in a vial of 10 ml of acetone and sonicated for 2 hours to achieve homogeneity. The solution was allowed to cool to room temperature before being spun into fibers.

4. Electrospinning of PMMA Solution:

Electrospinning was utilized to achieve a nanoporous scaffold of PMMA fibers. Figure 2 portrays the electrospinning apparatus. The parameters for electrospinning can be tuned in order to achieve uniformity and continuity and these parameters include: ambient temperature and humidity, viscosity of the solution, applied voltage, distance of needle tip to collecting plate, gauge of needle, and flow rate. The parameters used were: applied voltage 20 kV, separation distance of 18 cm, disposable needle gauge of 18, flow rate 5 ml/hr. PMMA fibers were dried overnight at 70° C which aids in transparency and thus light sensing applications [12]. Fibers were characterized using SEM and light microscopy.

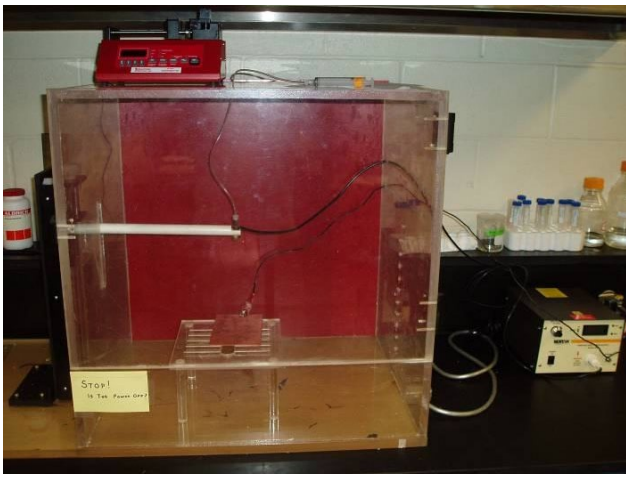

Figure 2: Electrospinning Apparatus

5. Functionalization of PMMA Fibers

A method proposed by Fixe [13] was utilized to functionalize PMMA fibers using hexamethylene diamine to create amine groups from the existing surface methyl groups[12]. First, nanofibers were cleaned by saturating the fibers with 95% isopropyl alcohol, just enough was micro-pipetted to cover the fibers, followed by a rinse with ddH_2O. PMMA is soluble in water, therefore the amount of ddH_2O to rinse the fibers had to be kept to a minimum. The fibers were then immersed in a 10% hexamethylene diamine/borate buffer (pH 11.5) solution for 2 hours, followed by two 15 minute washes of borate buffer pH 11.5 and pH 8.0 respectively. Finally, the fibers were left to dry at 30° C overnight and a Thermoscientific Nicolet 6700 FTIR with an ATR crystal was used to assess the surface functionalization.

6. Immobilization of Xerogel Sensor to PMMA fibers

Sulfo-GMBS was utilized as a cross-linker between functionalized (aminated) PMMA fibers and the MIPs. The thiol groups on the MIPs were cross-linked to the aminated PMMA fibers. The xerogel particles were immobilized on the PMMA fibers by submersion in 1 mM sulfo-GMBS in absolute ethanol solution for 1 hour followed by submersion in various concentrations of xerogel in absolute ethanol solution for 1 hour. The optimum concentration of xerogel will be experimentally determined in order to achieve a low limit of detection, but not saturate the optical detection equipment. SEM, optical microscope, and an Ocean Optics USB4000 spectrometer were utilized to determine immobilization of the fluorescent MIP particles to the PMMA electrospun fibers as well as the surface morphology.

FITC has a characteristic range of fluorescence when excited by light at 490nm wavelength that emits in the range of about 521nm. The optimum concentration was determined by varying the concentrations of MIP to ethanol and observing fluorescent peaks of seven points in the sample. The samples were recorded using Spectra Suite software with a 30 ms integration time and the fluorescence wavelengths of and peak intensities were recorded with the intention of using the smallest concentration that achieves a range of 10,000 to 20,000 counts. The sensitivity of sensor is directly proportional to the amount of MIP particles, for sensitivity is measured with the quenching of fluorescence by the analyte, therefore too much MIP particles makes a small change in fluorescence difficult to detect.

7. Optimizing Sensor Response to DNT

The response to DNT was measured by exposing the fluorescent MIP-PMMA fibers to different concentrations of DNT for 30 minutes. The sensor was exposed to an aqueous solution containing 0 to 1 mM DNT. After exposure, the DNT bound to the imprinted bindings sites, and the resulting decrease in fluorescence was recorded using the Ocean Optics USB4000 spectrometer attached to an Olympus IX70 inverted microscope (refer to Figure 3). A mercury lamp with a GFP filter cube excited the FITC. Ten regions on the PMMA fibers were scanned and the average drop in fluorescence upon DNT exposure was recorded. The data was analyzed by comparing the change in peak fluorescence intensity of each solution, and plotting these intensity values with respect to the concentration of DNT.

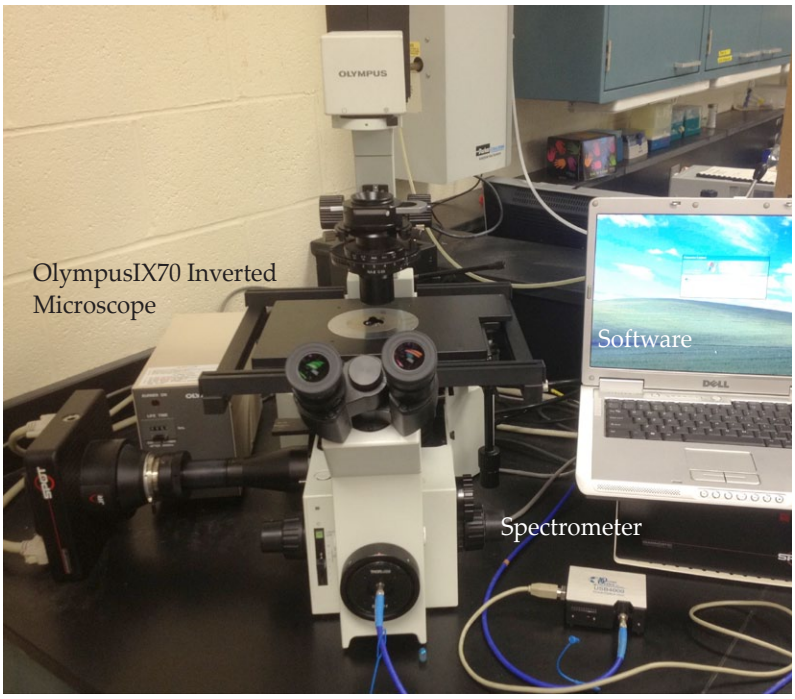

Figure 3: A photo of the optical detection system.

8. Dosing Response of Sensor

After determining the optimized concentration of MIPs to immobilize onto the PMMA fibers, a dosing response was conducted to determine the sensitivity of the sensor. All samples were immobilized with a 0.07 mg/ml MIP to ethanol concentration and various dilutions of DNT were formulated. The following concentrations of DNT to ethanol were used: 1mM, 0.1mM, 10 μ M, 1 μ M, 0.1 μ M, and a control of absolute ethanol. The samples fluorescence peaks were recorded with a 20x optical zoom to account for large standard deviations prior to exposure to DNT. Upon a 30-minute exposure to DNT, the difference in fluorescence intensities was recorded.

RESULTS/DISCUSSION

It was necessary to modify the surface of the electrospun PMMA fibers with amine functional groups. This created binding sites for the cross-linker, sulfo-GMBS, to bind the thiol groups of the MIP sensor to the amine groups of the electrospun fibers. Amine groups are characterized as peaks at 1550 cm^{-1} and 1650 $cm^{-1.}$. ATR-FTIR analysis (as shown in Figure 4, blue scan) provided confirmation that the functionalization protocol was viable by demonstrating peaks at 1552.1cm^{-1} and 1641.0cm^{-1}. The pristine fibers, shown in the red scan, clearly exhibit an absence of these characteristic peaks.

In Figure 5, SEM micrographs showed the subsequent development of the sensor and assessed the immobilization protocol. It is important to note that as the fibers were functionalized with the water washing, there were concerns with the morphological integrity of the fibers. Figures 2b and 2c demonstrates that after functionalization and immobilization, the fibrous and porous structure of the fibers were retained, as compared to the pristine electrospun fibers in Figure 2a. Further, the remaining images Figures 2d and 2e, confirmed immobilization of sensor particles on the electrospun scaffold at various concentrations. These figures show the heterogeneous distribution of the sensor particles on the scaffold. Larger concentrations of the MIP sensors tended to clump together, whereas lower concentrations tended to have better coverage along the scaffold. This heterogeneous distribution resulted in an unequal distribution of light intensity along the sensing scheme, which resulted in large variability in the spectroscopy data.

The fluorescence image, Figure 6, portrays the functionality of our sensing scheme. It further confirms that the MIP sensors are immobilized to the fibers, along with portraying the emittance of the fluorophore FITC in the visible green spectrum. Spectral analysis using Ocean Optics software and a USB4000 spectrometer confirmed a fluorescent peak at ~525nm range, indicating adequate MIP sensor function and signal. Also, Figure 6 portrays a very large concentration of MIPs to the fiber that might overwhelm the signal and result in self-quenching. It would be imperative to perform future studies on the ideal concentration of MIP sensors immobilized on the fibers. Unfortunately, there were large variability's in light intensity along the electrospun fibers due to heterogeneous binding of the MIP sensors. Some points are brighter or dimmer than others, and upon conducting fluorescence spectroscopy measurements to determine the optimal concentration of sensor to fiber, there were large standard deviations in the measurements.

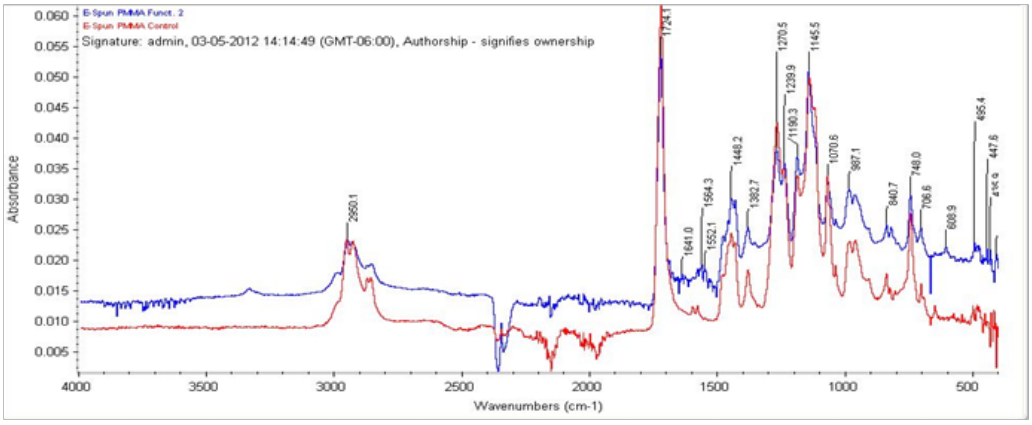

Figure 4: ATR-FTIR spectrum of unfunctionalized (red) and functionalized (blue) PMMA fibers.

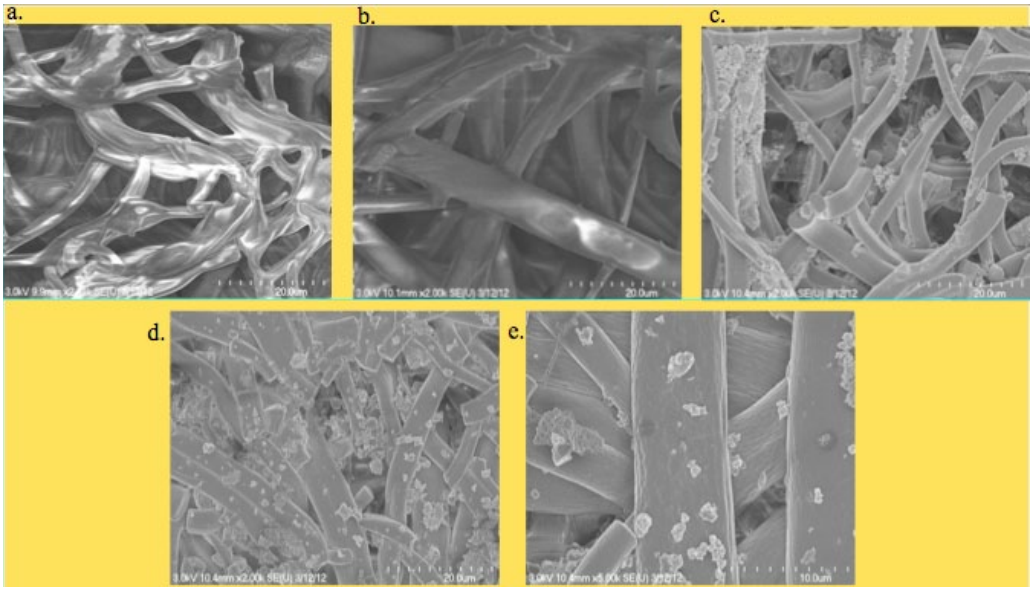

Figure 5: Micrographs of PMMA fibers at various stages of development process: a) Pristine PMMA fibers; b) Functionalized PMMA fibers; c) Immobilized xerogel to PMMA fibers at a concentration of 0.1 mg/ml (MIPs to ethanol); d) Immobilized xerogel to PMMA fibers at a concentration of 10 mg/ml (MIPs to ethanol); e) Immobilized xerogel to PMMA (conc. 10 mg/mL) at 5000x zoom.

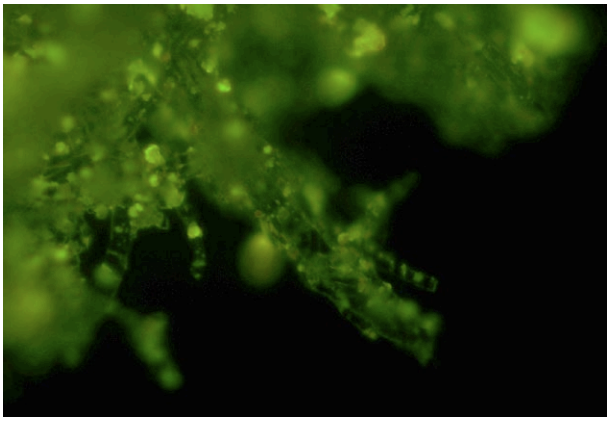

Figure 6: Fluorescence image of MIP sensor immobilized onto electrospun PMMA fibers.

Figure 7 displays the change in fluorescence intensity after DNT exposure for different concentrations of MIPs immobilized to the electrospun fibers. The results show large standard deviations and there were no significant differences between concentrations. However, an optimal concentration may be the 0.07 mg/mL (MIPs/ethanol) since it portrayed the largest change in fluorescence intensity while maintain a low concentration. Therefore, we theorized that this concentration would be pursued because it would provide a range of detection that could measure small changes in intensity while avoiding oversaturating or under saturating the optical detection equipment. It is important to note that the control (no MIPs) also displayed a very large change in fluorescence intensity, but this is to be attributed to contamination in the sample. The forceps were not washed after handling a prior sample that had immobilized MIP sensors and thus some MIPs were entrapped. Concentrations above 0.1 mg/mL were omitted from the study because they oversaturated the detection equipment.

Next, the optimal concentration (the 0.07 mg/mL (MIPs/ethanol)) was utilized to assess the dose response and sensitivity of the MIPs sensor upon exposure to DNT. Various concentrations of DNT from 0.1 μM to 1 mM were dosed on the fibers and changes in fluorescence intensity were measured. Figure 8 displays the results. Prior research has shown that the MIP thin film sensors can be sensitive in the parts per billion (ppb) range [3]. Our results indicate that changes in fluorescence up to 0.1 μ M could be observed, but this data was not significant because of tlarge standard deviations. These deviations further indicate the variability in the light distribution along the electrospun fibers and that the technique utilized to immobilized the MIPs and measure light intensity can be improved. There was difficulty in accurately reproducing exact sample points along the electrospun fibers with the inverted microscope. Further, some of the sensor particles were likely not immobilized, but rather mechanically constrained by the fibers, which, upon DNT dosing, shifted on the substrate or were washed away.

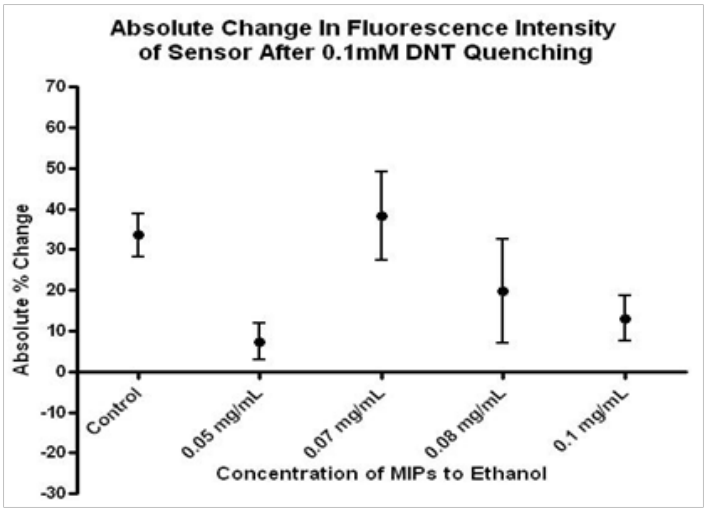

Figure 7: Absolute Change in Fluorescence intensity upon 0.1 mM DNT dosing of various concentrations of immobilized MIP sensor on electrospun fibers.

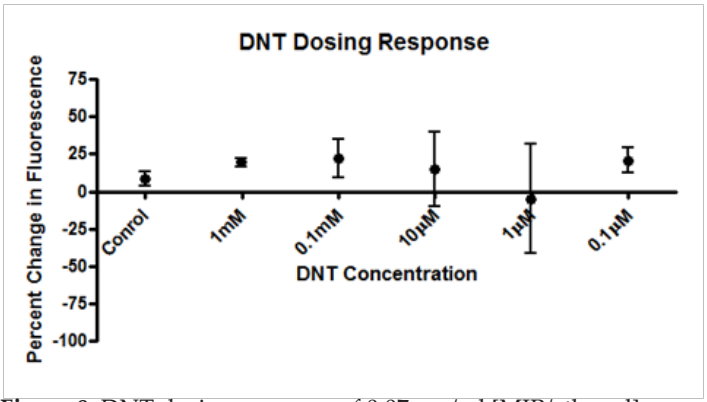

Figure 8: DNT dosing response of 0.07 mg/ml [MIP/ethanol] immobilization on electrospun fibers.

CONCLUSION

The result gathered sufficient preliminary data on a novel sensing scheme. Molecularly imprinted polymer conjugates were successfully immobilized on electrospun PMMA scaffolds, and the sensor responded to changes in DNT concentrations. The data gave insight as to the limitations of the methods; specifically with functionalization, immobilization, and data acquisition. Functionalizing and immobilizing the fibers were difficult due to the fact that the fibers would partially dissolve, causing the substrate to be brittle, and/or gelation of the substrate. A few attempts at creating the sensor succeeded, but it was difficult to reproduce. The heterogeneous distribution of the MIP sensors on the electrospun scaffolds resulted in large variations of light intensity along the fibers. This variation created large standard deviations in fluorescence measurements, and implies that the sensing scheme can be improved by attempting to homogeneously distribute the sensor on the scaffold. This could be accomplished by attempting to dip coat the fibers in the MIP solutions and having the xerogel form around the fibers. This has the added benefit of avoiding the harsh chemical treatments necessary to functionalize the fibers and immobilize the sensor. Future work will look at improving this sensing scheme.

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Effects of previous experience on aggressive behavior in male gray treefrogs, *Hyla versicolor*

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ABSTRACT

Aggressive behavior has been studied intensively in animals because the factors involved in success in aggressive interactions may be a major component of individual fitness. An individual's past experience could affect its behavior in future aggressive interactions. In fact, studies of many animal taxa have shown that previous aggressive experience can alter an individual's behavior in future aggressive interactions. Frogs have long been a model for studying acoustic communication, which includes signals used in aggressive encounters. We tested the hypothesis that the aggressive behavior of male gray tree frogs (*Hyla versicolor*) that have had experience in competitive interactions differs from that of males maintained in relative social isolation. We also tested the hypothesis that winners of prior interactions will continue winning future interactions (winner effects) and losers will continue losing (loser effects). We tested these hypotheses by staging aggressive interactions between males with past experience and isolated males. We compared the likelihood of winning, the escalation and the duration of interactions between males with and without different levels of aggressive experience. Although further data collection is needed, our data suggest that an individual's past experience may influence the characteristics of future aggressive interactions. Specifically, we found that individuals that were more socially isolated engaged in longer-duration interactions than socialized males. The duration of a previous interaction was also negatively related to the duration of a subsequent interaction, but only for winners. We did not, however, find any evidence of winner and loser effects.

INTRODUCTION

All living organisms share the world's complex ecosystems and are therefore always interacting with one another. Communication is a vital component of these constant interactions between organisms (Bradbury & Vehrencamp 2011). Anurans (frogs and toads) have always been important subjects in the study of animal communication (reviewed by Gerhardt & Huber 2002). Much of the focus has been on female choice of mates, but other important aspects of communication have received less attention in anurans. I will discuss what is known about communication in anurans and in particular I will focus on a type of communication about which little is known for this group, aggressive signaling between males.

Role of Communication in Anurans:

Animals communicate in a number of ways including: acoustic, visual, chemical and tactile (Bradbury & Vehrencamp 2011). The modality of communication is highly dependent

on the life history of the organism. In particular, reproduction is highly dependent on communication, whether it is between males or males and females. For example, the reproductive behavior of gray treefrogs primarily involves acoustic communication because mating occurs at night where visual communication is less efficient. Reproduction is a major component of fitness, and therefore natural and sexual selection have exerted a strong influence on communication in this context.

Some anurans may use a combination of more than one form of communication, which is known as multimodal communication (Richardson et al. 2010). For example, some anuran species utilize both visual and acoustic communication (Hödl & Amezquita 2001). Sexual selection, the concept of individuals struggling for mating opportunity, has had a particularly strong impact because it affects communication involved in both male-male competition and female choice (Darwin 1859; Andersson 1994). Female choice requires a display from conspecific males. Males in many species exhibit lek behavior in which males gather and display to attract females, who may be choosy and select certain males as mates (Wiley 1991). For many anurans, advertisement calls are displayed within a chorus setting where males gather and call to attract females (Wells 1977). A chorus typically contains calls from males of many different species, whose calls are species-specific. This lek breeding system causes competition for mates to be high. In typical anuran leks, each male defends a small space in order to avoid interference from other calling males (Wells 1977). Clearly, competition among males can be very tense because of the high density of males relative to the number of females and the importance of reproduction to male fitness.

In response to the density of the chorus and changes in competition, males can alter call characteristics such as call complexity, call duration, call rate, call effort and call frequency (Wells 1988). Many species of anurans generate simple calls that contain one note type, whereas others may give more complex calls, which consist of multiple note types (Larson 2004). For example, in dense choruses, males of some species increase their call complexity, which makes them more attractive relative to other males within the chorus (Rand & Ryan 1981). To avoid acoustic interference and improve attractiveness to females, males in close contact may alternate calls (Schwartz 1987). Although the lek behavior creates a very competitive environment, it demonstrates the ability of individuals to adapt to various strenuous situations concerning their overall fitness. Males communicate with one another using several different types of calls.

Males use a series of advertisement calls mainly to attract conspecific females, but these signals may also be used in male-male competition as well (Wells 1977). Call characteristics are often different between different species, and therefore advertisement calls are important in preventing mating among the wrong species. If a rival male encroaches on an individual's call space, a male may give an aggressive signal during disputes over calling spaces (Duellmann & Trueb 1986). Males also give release calls if another male attempts to mate with him or he is held by humans (Duellmann & Trueb 1986). In addition, when threatened by predation, many give distress calls (Duellmann & Trueb 1986). Males are often the only ones that vocalize within a species, but this is not always the case. Although rare, females may respond to male calling with a reciprocation call (Marquez & Verell 1991). This behavior allows a female to better communicate with her mate. Cleary, frogs communicate with one another in numerous different ways, all of which are important to their overall fitness. Yet, an aspect of communication that is equally important and yet understudied is that of aggressive signaling. Therefore, I will focus the remainder of this paper on the aggressive calls that appear to be involved in deterring rival males (Wells 1977).

Aggressive Behavior:

Aggressive behavior is a key component to an organism's life history. In many species, aggressive interactions are what determine territory ownership, mating opportunity, and even necessities of life such as food and water (Clutton-Brock et al. 1979). Aggressive interactions, specifically physical fighting can be very costly to an individual (Clutton-Brock et al. 1979). Therefore, it is often more beneficial to resolve a conflict through aggressive signaling rather than physical fighting (Maynard Smith and Parker 1976). Physical fighting is therefore often rare compared to signaling in many anurans (Howard 1978, Fellers 1979, Crump 1988). With this in mind, it is also clear why males are typically less willing to fight a male that he may not be able to beat (Clutton-Brock et al. 1979). Clearly, aggressive behavior can have major impacts on an individual's fitness.

Aggressive behavior has been studied in great detail in many taxa but not much is known about aggressive behavior in frogs. When tested using playbacks of calls of different intensities, male Blanchard's cricket frogs, *Acris crepitans blanchardi*, became more aggressive, giving graded aggressive calls that signaled changes in the probability of attack as the playback level of the sound got louder, simulating an approaching rival. They also responded more aggressively to low-frequency playbacks since low-frequency calls typically correlate to larger, more competitive, males (Wagner 1989). Wells and Schwartz obtained similar findings in males of the neotropical treefrog, *Hyla ebraccata* (Wells & Schwartz 1984). These males responded aggressively to both advertisement and aggressive playbacks. These authors also found that pulse repetition rate and rise time are very important in eliciting aggressive calling (Wells & Schwartz 1984). More recent research on aggressive behavior has examined the presence of a "plastic" aggressive threshold, which is the level at which males tolerate the presence of other males. Tests among males suggest that this threshold is malleable and is subject to habituation, defined as waning of responsiveness caused by repeated stimulation(Reichert 2010). Understanding chorus formation as a whole is vital to understanding aggressive behavior and how it is elicited. While the incidence of aggressive calling within a chorus is high in the early evening while males are setting up temporary territories, production of aggressive calls typically wanes by late evening when a stable chorus has become established. Additional playback studies suggest that male, *Hyla ebraccata*, are sensitive to both the number of aggressive calls and the call characteristics of those aggressive calls, which could impact the outcome of future interactions (Reichert 2011). Although some studies such as those just discussed, have examined aggressive calling behavior, we still

know very little about how aggressive calls actually function to resolve aggressive interactions between males (Wells et al. 2007). Because frogs are such important research subjects for the study of animal communication, it is important to learn more about how their aggressive communication system works.

Effects of Previous Experience:

One particular aspect of aggressive behavior that has been studied in other taxa but neglected in frogs is that of previous contest experience (e.g., Rutte et al. 2006). Many studies show that previous contest experience affects the outcome of future contests (Hsu et al. 2005). Some particularly interesting effects of previous experience are winner and loser effects. In these phenomena, individuals that have recently won a competition are often more likely to win future competitions (winner effects), and individuals that have recently lost a competition are often more likely to lose future competitions (loser effects) (reviewed by Chase et al. 1994). Previous studies have shown the existence of winner and loser effects in many species of insects, birds, reptiles, mammals and fish, but this phenomenon has never been investigated in frogs (Hsu et al. 2006). Little is known about what causes these effects. One hypothesis, the social-cue hypothesis, suggests that winning or losing one competition may leave traces and alter an individuals' response to subsequent opponents. The other, the self-assessment hypothesis, suggests that the individual may gain more information about his own fighting ability and in turn alter his response to subsequent opponents (Rutte et al. 2006). Previous experience appears to be an important aspect of aggressive interactions in animals, so it is important to know if these effects occur in anuran contests.

Summary:

Clearly, communication is vital because animals constantly interact with other individuals. As an important subject used in behavioral ecology, it is necessary that we understand all aspects of communication among frogs. Although aggressive signaling is a major aspect of communication, it is understudied among frogs. Like many other taxa, competition among male frogs is influenced by social experience (Oliveira et al. 2009).

Competition among males within a chorus setting is high and therefore aggressive signaling may be an important component of reproductive success. More specifically, winner and loser effects, which have been studied in other taxa, have not been studied in frogs and therefore our understanding of what alters aggressive interaction is lacking as well. With this in mind, my study of the effects of previous experience in the gray treefrog, *Hyla versicolor*, will add to our understanding of how previous experiences alters the behaviors within a contest as well as the outcome of a contest.

METHODS

We staged aggressive interactions during the summers of 2010 and 2011. Males were collected from natural populations in ponds near Ashland, Mo and transported to a greenhouse facility in Columbia, Mo. During the period of testing individuals were housed for up to one week and given food and water ad libitum. On each day of testing, males were released into the artificial pond, an octagonal enclosure with sides 2 m in length, containing about 3 inches of water, every afternoon (details in Schwartz et al. 2001). The number of males collected for experimentation depended on climate, and therefore the number of males released into the artificial pond was variable. To encourage males to call, we simulated an afternoon rainstorm using overhead sprinklers and played back a digital version of an artificial chorus from an overhead sound system. Testing took place after sunset and lasted until the chorus subsided. Because testing took place at night, all tests were done in the dark. After a male was used in a test, it was toeclipped for individual identification and then returned to its natural population at the Baskett Natural Area near Ashland, Mo.

Experiment 1: Winner and Loser Effects

We tested whether winner and loser effects are present in *H. versicolor* during May-July of 2011. To ensure that there were no differences between males in previous aggressive experience, we performed these tests on 'isolated' males. Isolated males were caged to prevent interaction with other males prior to testing. Because body size often plays a role in aggressive interactions, males of the same body mass were selected to control for this variable. Sixteen sized-matched males were placed directly on Styrofoam platforms and covered with a mesh enclosure as well as an additional (.4445 m high, .508 m in length and .3429 m in width) wooden cage. The buffer zone between the large wooden cage and smaller mesh cage prevented any physical contact or aggressive interactions between the isolated male and other males in the pond, but the cages were visually and acoustically transparent. In addition to the 16 isolated males, we released other males that could move freely throughout the pond. These males were used for other experiments, and their presence increased the likelihood that the isolated males would call. The males we used as 'isolated' males for any given night may have been among those males swimming freely in the artificial or natural pond on previous nights, and thus they could have been involved in aggressive interactions on those nights. We considered it unlikely that effects of previous experience would carry over between nights because the memory capacity of lekking frogs is probably low (Akre & Ryan 2010). Isolated males that called within the cages were removed and transferred to the testing arena, located approximately 3 m away from the artificial pond (see below).

We tested for winner and loser effects in two steps (Figure 1A). First, two calling males were selected from the sixteen isolated males and transferred to the testing arena. We staged an aggressive interaction between these two males (see below). This initial aggressive interaction produced an 'initial winner' and an 'initial loser'. Second, we paired both the initial winner and the initial loser against naïve males (other calling, isolated males that had not previously participated in an interaction; see Figure 1B). The order in which we retested the initial winner and loser was determined randomly by a coin flip, although this order was not always followed because some males from the initial interaction failed to resume calling immediately. As in the initial interaction, we noted the winner and loser of each of the interactions (initial winner vs. naïve male 1 and initial loser vs. naïve male 2).

Staged interaction procedure:

We staged interactions between males using the procedure

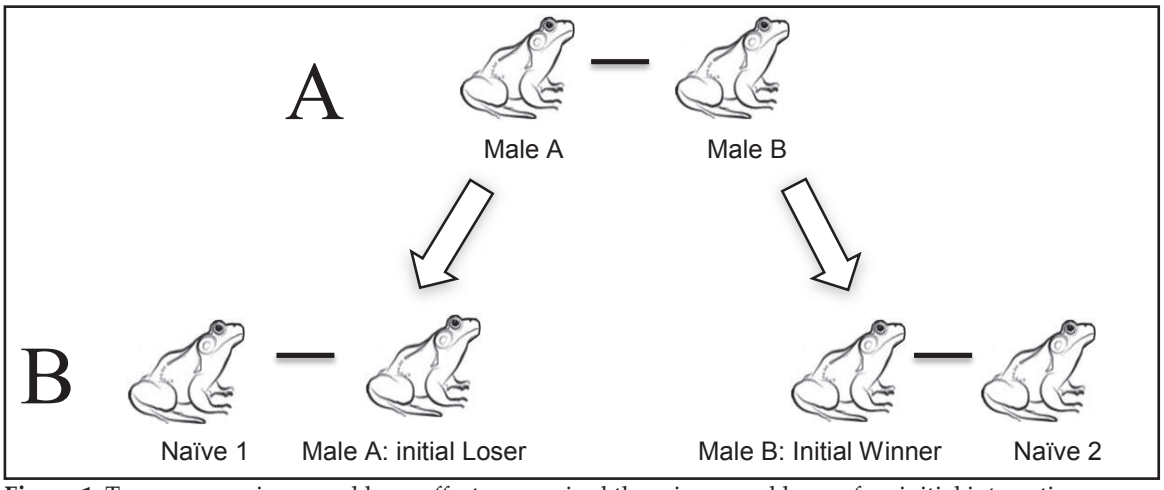

Figure 1: To measure winner and loser effects, we paired the winner and loser of an initial interaction (Figure 1A) against naïve males (Figure 1B).

described in Reichert & Gerhardt (2011). The testing arena consisted of a wooden runway (1.8 m in length, 0.3 m in width) and two wooden wheeled platforms surrounded by an opaque mesh to prevent males from escaping or being distracted. Calling males were carried atop their Styrofoam platform from the artificial pond to the testing arena, where each male was placed on a platform on opposite ends of the arena about 0.9 m apart. We suspended a directional microphone (Sennheiser ME-66) directly above each of the males. After males began calling on the platform, the mesh lid was removed. We recorded ten baseline calls from each male onto a Marantz PMD-661 digital audio recorder (44.1 KHz sampling rate, 16-bit digital PCM files). After this baseline period, we pulled the platforms gently using ropes attached to the platforms. Once the platforms were adjacent to one another, aggressive interactions often ensued. We video recorded the interaction using the night vision setting on a Sony DCR-SR85 camcorder.

We defined an interaction as having taken place when both males gave at least one call when the platforms were adjacent to one another. Interactions were allowed to continue until there was a clear winner and loser. A loser was defined as the individual that stopped calling for at least five minutes or escaped the interaction. After the interaction took place, we noted the time of night and measured the mass, snout-vent length (SVL), and temperature of both the winner and loser.

Data analysis:

The hypothesis that winner and loser effects take place in this species predicts that initial winners would be more likely to win subsequent interactions against naïve males than initial losers. Initial losers would be less likely to win subsequent interactions against naïve males. Thus, we calculated the proportion of previous winners that won again and of the previous losers that lost again. We compared this proportion to the null expectation that initial winners and losers would be equally likely to win the subsequent interaction and tested this result with a two-tailed binomial test.

We were interested in whether previous winners and losers would differ in their behavior in subsequent interactions. Thus, we compared behavioral measures of initial winners and loser during their initial and subsequent interactions. The behaviors considered were the level of escalation and duration of each individual's initial and subsequent interaction. Levels of escalation are defined as follows. Within any given interaction, the lowest level of escalation is the production of advertisement calls only (adv calls only). At the next level, one male produces an aggressive call (one way agg), and at the third level, both males produce aggressive calls (two way agg). At the highest level of escalation, a physical fight occurs. Any physical contact within an interaction was classified as a fight.

The duration was the length of the interaction in seconds. Specifically, we measured the interaction duration, which is the time from the beginning of the interaction to the last call. We used paired statistical tests to examine changes within and between males in each behavior.

Experiment 2: Effects of social isolation on aggressive behavior

We tested whether aggressive interactions differed when they were staged between pairs of males that were considered to either have had previous experience in aggressive interactions ('experienced' males) or males that had no previous experience in aggressive interactions ('isolated males'). Data for interactions between isolated males were taken from the 'initial' interactions of our winner and loser effects experiment, described above. These interactions involved two males that had no previous experience engaging in aggressive interactions on the night of testing. Data for interactions between experienced males were taken from experiments performed by Reichert & Gerhardt (2011). In these experiments, interactions were staged between males that were allowed to move freely about the pond. Although the exact nature of the males' previous experience in these experiments is unknown, the high density of males placed in the artificial pond made it likely that each individual experienced one or more aggressive interactions (M.S. Reichert, personal communication). Thus, males we termed as experienced were likely to have engaged in previous aggressive interactions prior to testing. Other than ability of experienced males to move freely throughout the pond, the testing procedure for experienced males was identical to that described above for isolated males.

RESULTS

Experiment 1: Winner and loser effects

We instigated twenty-four interactions between isolated males. There were no apparent differences between the weights or lengths of males in these competitions (Mean ± SE for initial winner mass: 6.40±0.46 g, initial loser mass: 6.38 ±0.40 g, initial winner length: 46.7±2.9 mm, initial loser length: 47.0±3.1mm). We also examined the interactions of fifteen initial winners against naïve individuals and ten initial losers against naïve individuals. There was also no evidence of differences between the weights and lengths of the naïve individuals that were paired with initial winners and losers (Mean ± SE for mass of naïve individuals paired with initial losers: 6.52±0.41g (N=16), length of naïve individuals paired with initial losers: 47.1±2.5mm, mass of naïve individuals paired with initial losers: 6.53±0.38g (N=10), or length of naïve individuals that faced losers: 48.0±1.8mm. There was no evidence for winner effects (Figure 1: binomial test, N=15, P=0.302) or loser effects (Figure 1: binomial test N=10, P=1)

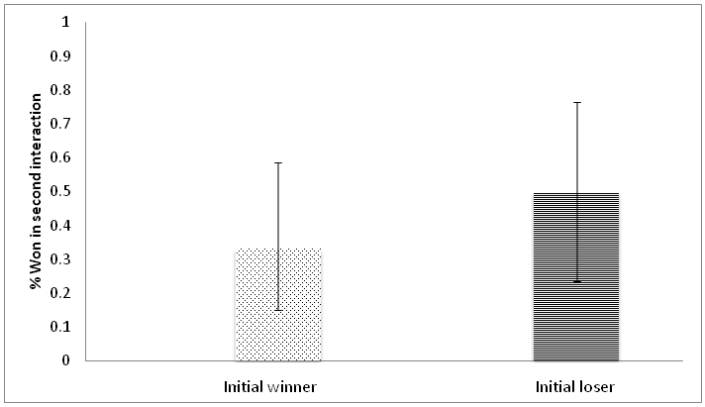

Figure 1: Proportion of the second interactions won (N=25 total interactions) by initial winners and initial losers. Winner effects would be indicated by initial winners winning a significantly greater proportion of their second interactions than the random expectation of 50%, while loser effects would be indicated by initial losers losing a significantly greater proportion of their interactions than the random expectation of 50%. We found no significant evidence for either winner effects or loser effects.

We compared several characteristics of the interactions between males in their initial interaction and in their subsequent interaction vs. a naïve male to determine if previous experience in aggressive interactions had effects on subsequent interactions. First, we examined the durations of initial and subsequent interactions. For winners, there was a significant negative relationship between the duration of their first interaction and the duration of the second interaction (Figure 2: N=13, R^2=0.55, P=0.0004). However, for losers, there was not a relationship between the duration of the initial interaction and the duration of the second interaction (N=9, R^2=0.03, P=0.65).

We also compared the level of escalation of the initial interactions to that of the subsequent interaction for both the initial winners and initial losers. We first categorized the levels of escalation into four categories as defined above. There appeared to be no differences between the affects of each treatment group on level of escalation (Figure 3).

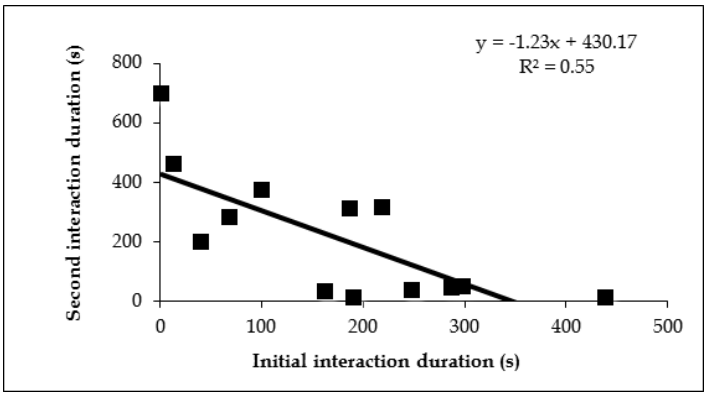

Figure 2: Scatterplot showing the relationship between the duration of the initial interaction and the subsequent interaction for individuals that won their initial interaction. Trendline is the linear least-squares regression line (y=1.23x + 430.17).

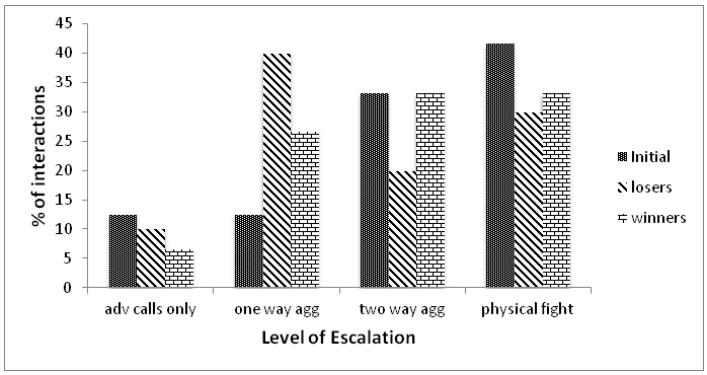

Figure 3: The percent of interactions that reached the given level of escalation for the initial interaction (N=24), initial winners vs. naïve (N=15) and initial losers vs. naïve interactions (N=10).

We also ascertained whether the individual that was the aggressive instigator in the initial interaction was also the aggressive instigator in the second interaction. The aggressive instigator is defined as the male that first initiates an aggressive interaction, either by use an aggressive call or physical contact. We found that individuals tended to be consistent in their behavior in both interactions (chi-square test; winners: chi-square = 4.7, DF=1, P=0.031, for losers: chi-square=2.0, DF=1, P=0.16 (Table 1).

		Second interaction	
		Naive	Initial Winner
Initial interaction	Loser	6	2
	Winner	1	5

Table 1: Individuals tended to be consistent in their behavior in both interactions. 78% of the time, the initial winner behaved the same in the initial interaction and the second interaction.

This consistency in behavior has been of recent interest in the literature. Males that behaved one way in the initial interaction repeated their behavior in subsequent interactions. This is specifically of interest since other data suggest that ectotherms are less likely to repeat behavior since they are highly dependent on the environment (Mousseau et al 1987). Repeatability is highly dependent on the type of behavior being examined. Because

aggressive behavior is controlled by testosterone, it is often more predictable than other behaviors (Andrew 1972, Winfield 1994). Therefore, males that behaved aggressively in the initial interaction behaved similarly in the second interaction.

Experiment 2: Isolated vs. Experienced Males

We compared the interactions of twenty-four isolated males and fifty-two experienced males to determine if previous experience had an effect on aggressive behavior. We compared the level of escalation between the two treatment groups and found no evidence that previous experience altered their propensity to escalate in contests (Figure 4).

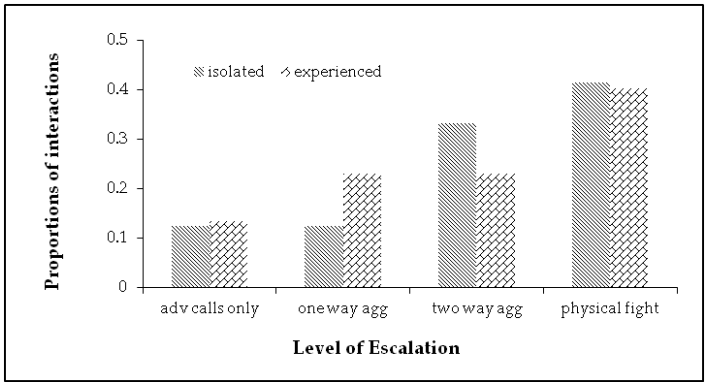

Figure 4: No difference between the level of escalation of interactions involving isolated (N=24) or experienced males (N=52).

We also compared the interaction duration of males with previous experience vs. isolated males. The duration of interactions between isolated males was significantly longer than interactions between experienced males (Figure 5: Wilcoxon-Mann-Witney test: N=72, Z=2.05, P=0.04).

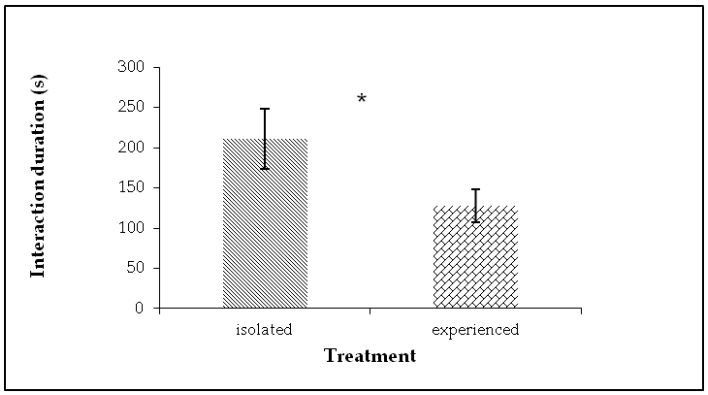

Figure 5: Interaction duration of contests between isolated males was significantly longer than that of contests between experienced males.

DISCUSSION

Winner and Loser Effects:

Winner and loser effects have been documented in many taxa including insects, birds, reptiles, mammals and fish (Oliveira et al. 2009). This study is one of the first to study this phenomenon in frogs, and we predicted that winner and loser effects would take place in this group because they are so common in other taxa. Surprisingly, we did not find evidence of winner effects or loser effects in gray treefrogs. In fact, there was a trend for initial winners to win less often than expected in subsequent interactions. Participation in contests can be very energetically costly and exhausting, and this may be especially true for contest winners (Briffa et al. 2007). Therefore, winners of initial interactions may not have enough energy to continue winning future interactions without recovery time. However, too much recovery time can lead to the decay of winner and loser effects (Bakker et al. 1989, Chase 1994, Hsu et al. 1999). We also found a significant negative relationship between duration of the initial interaction and the duration of the subsequent interaction for winners. In other words, the longer the initial interaction, the shorter the second interaction. This could also be due to the fact that aggressive interactions are energetically expensive and males are unable to continue expending energy on subsequent interactions.

Previous studies also found that winners of initial interactions were more likely to exhibit a higher level of escalation during the subsequent interaction (Hsu et al. 2007). We did not find any differences in the level of escalation between the initial interactions, loser retests or winner retests.

Therefore males may avoid aggressive interactions completely in order to save energy for attracting females or may limit the number of aggressive interactions since they are often energetically costly (Briffa et al. 2007). This may explain why males often spread out and alternate calls in their natural setting. (Wells et al. 2007).

Effects of Previous Experience on Aggressive Behavior

We expected to find significant differences in the aggressive behavior of isolated males compared to experienced males. We did not find a difference in the level of escalation of interactions involving isolated males compared with experienced males. Thus, the intensity of interactions between two isolated males or two experienced males was very similar. Previous studies have suggested that males with previous experience in aggressive interactions are often more aggressive in subsequent interactions but it has also been suggested that the energy expended in aggressive interactions can exhaust males and limit their ability to compete in subsequent interactions (Hsu et al. 2007).

Although previous experience did not have an effect on contest escalation, experience did affect the duration of interactions. Isolated males had significantly longer interactions than experienced males. One possibility is that experienced males had more social experience and were more habituated (Marshall et al. 2003, Brenowitz et al. 1994). The duration of contests could also be affected by the characteristics of the previous interaction.

Summary

In conclusion, more research is needed to better understand the effects of previous experience on aggressive behavior, specifically winner and loser effects. Although we did find evidence that previous experience affected the duration of interactions, and not the escalation, further research is needed to explain this pattern. In addition, more data collection for winner and loser effects is imperative for testing this phenomenon in gray treefrogs.

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Parasitism of Hatchery Rainbow Trout By *Salminocola Californiesis*: Attachment Sites, and The Ontogeny and Ecology of Infection

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ABSTRACT

Ectoparasitic copepods of salmonids attach to the body surface and branchial cavity. Extensive parasitism of an individual host can result in morbidity and decreased probability of survival. A species specific copepod, *Salmincola californiensis* parasitizes several species of trout, most notably rainbow trout (*O. mykiss).* This study was conducted to document the ontogeny of the parasite-host interaction by recording how newly hatched, uninfected fish that are released in an infected hatchery develop their parasite infrapopulations (that is, the parasite population of a single fish) and component population (the parasite population of an entire population of hosts). The study was conducted at the Maramec Spring Trout Hatchery, St. James, MO. Individual fish were surveyed biweekly to determine the number of viable copepods present on the animal. We found that there was no significant asymmetries between the left and right gills of the individual and host weight may impact the copepod abundance.

INTRODUCTION

Ectoparasitic copepods of salmonids attach to the body surface and gill filaments. Extensive parasitism of an individual host can result in morbidity and decreased probability of survival. In natural settings the prevalence of highly infected individuals is typically low. In aquaculture settings, however, the prevalence and intensity of parasitism can be much higher, with associated economic costs due to morbidity, treatment, and quarantine practices (Piasecki et al. 2004). While copepod-host dynamics have received considerable attention for marine pen-reared salmon, where primary copepods of concern are *Lepeopitheirus* and *Caligus* spp. (Siphonostomatoida; Caligidae), our understanding of copepod interactions with freshwater trout species remains underdeveloped. A number of studies have documented the extent of parasitism by *Salmincola* (Siphonostomatoida; Lernaeopodidae) copepods on freshwater *Oncorhynchus, Salmo,* and *Salvelinus* spp salmonids in field (Black et al. 1983; Chigbu 2001; Barndt & Stone 2003; Muzzall 2007) and hatchery settings (Sutherland and Wittrock 1985; Modin and Veek 2002). The later studies are particularly notable in demonstrating that parasitism by *Salmincola* can be a serious concern in trout farms.

Salmincola californiensis parasitizes several types of trout, most notably rainbow trout (*O. mykiss).* This copepod has a life cycle in which a free-swimming copepodid must find a host within two days of hatching, but then remains on that host for the remainder of its life (Kabata and Cousens 1973). It is uncertain how *S. californiensis* chooses a host, but research on brook trout (*Salvelinus fontinalis*) parasitized by

Salmincola edwardsii suggests that the copepodids find potential hosts by detecting shock waves and visual cues of passing fish (Poulin et al. 1990). Upon reaching a host, the *S. californiensis* parasite migrates to the fins or branchial cavity and attaches (Kabata and Cousens 1973, 1977). No apparent preference exists for a particular gill or side of the body (Chigbu 2001; Barndt and Stone 2003). It is unclear, however, if an initial infection enhances the likelihood of further infection of a gill, perhaps due to damage to some part of the gill that in turn interferes with gill or operculum function. Infections can result in hyperplasia and atrophy or growth inhibition of affected gill filaments and reduced reproductive performance (Gall et al. 1972; Kabata and Cousens 1977; Sutherland and Wittrock 1985; Roberts et al. 2004). While the overall distribution of parasitism by *S. californiensis* is skewed such that most hosts harbor few parasites while a few harbor most parasites, the factors intrinsic to the host that underpin individual-level infections are largely unknown. Host size, age, and sex may play a role (Chingbu 2001; Barndt & Stone 2003). However these factors have not been well studied for *S. californiensis* infection.

As part of a broader effort to address this parasite-host interaction in a hatchery setting where *S. californiensis* has caused significant difficulties for the management of the hatchery, we examined the spatial and temporal variation in the site of copepod attachment on hatchery-reared rainbow trout. Our goal was to use longitudinal sampling of a group of marked fish to document the ontogeny of the parasite-host interaction by recording how uninfected fingerling trout that are released in an infected hatchery develop their parasite infrapopulations (that is, the parasite population of a single fish) and component population (the parasite population of an entire population of hosts). We were particularly interested in three aspects of the development of a parasite-host interaction: 1) temporal variance in parasite abundance and aggregation of known individuals; 2) location of initial infection and the possibility that infection at a particular location begets further infection at the same location; and 3) the role that host size may play in the extent of parasitism.

METHODS

Field work took place at Maramec Spring Hatchery in Maramec Spring Park, St. James, Missouri. The hatchery is managed by the Missouri Department of Conservation, while the park is privately administered by the James Foundation. The hatchery is fed by Maramec Spring, the fifth largest spring in Missouri, with average daily flows of approximately 100 million gallons. The spring opens beneath a pool, which in turn flows to the Meramec River (Fig. 1). This pool contains a resident rainbow trout population that is infected with *S. californiensis*. The size, depth, and flow rate make treatment or eradication of either the trout or copepod from the spring pool impractical. Water diversions from the spring pool feed the raceway and several pools that act as the Maramec Spring Hatchery. However, water redirected from the spring also facilitates infection of trout raised in the pools.

Data collection took place from mid-June through mid-December 2011 from tagged, caged fish raised in two of the hatchery's serial raceway pools (Fig. 1). Three cages (ca 1m x 1m x 2m) were placed in Pool 1, which contained an infected

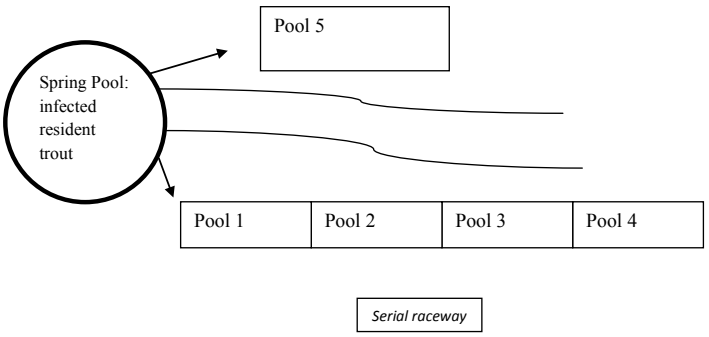

Figure 1. Schematic of Maramec Spring Trout Hatchery and Maramec Spring.

population of 2 yr old trout (mean length and wt in June 2011: 492 mm; 1511 g). A total of 60 of these trout were tagged and placed in two cages (30 fish/cage) to facilitate regular assessment of background rates of parasitism at the hatchery. These fish were sexed based on morphological features. A third cage contained 135 <1 yr old tagged trout obtained from an uninfected hatchery (Montauk Hatchery, Salem, MO; mean length and wt in June 2011: 184.4 mm; 56.7 g). Uninfected <1 yr old trout were also placed in Pool 3, along with one cage of 100 tagged, uninfected trout. During the course of this study, Pool 2 was stocked with brook trout in an effort to determine whether the filtering of water through a brook trout population might facilitate removal of parasitic copepodids and an associated decline in parasitism (Modin and Veek 2002). However, data for these fish (n = 235) are combined for all analyses. Young trout (<1 yr old) were not sexed for the analysis presented here.

Counts of the number of copepods on each fish were conducted every 2-3 wks by a team of 2-3 people. Cages were temporarily partitioned to separate processed and unprocessed fish. Trout were placed in an 80-135 mg/liter solution of MS-222 (tricaine methansulfonate), an FDA approved anesthetic, for approximately 45 seconds, then weighed, measured, and examined for attached copepods. For each fish the left and right gill and operculum as well as the mouth and body surface were recorded and the number of attached copepods on or immediately adjacent to each body part were recorded. Processing of each fish took 20-30 seconds, after which the fish was returned to the cage where equilibrium was regained within 3-5 min.

Following definitions of Bush et al. (1997), for each body region (mouth, left gill, right gill, total) we quantified prevalence (percent of examined fish harboring parasites), and abundance (number of parasites on a host). To document the ontogeny of the parasite-host interaction we calculated each measure at each sampling interval, contrasting those measures derived from newly introduced (i.e. unparasitized) individuals with measures calculated from the older Pool 1 individuals that had been exposed to the parasites for >2 yrs.

Based on total counts of copepods for each fish, we examined relationships between prevalence and sampling interval, host weight and length (>2 yr old fish only). We assessed for correlations in the abundance of copepods parasitizing different regions of the same fish using Wilcoxon signed-rank tests for left versus right gills and for gills (left and right combined)

versus mouth. We also assessed whether infection at a particular location increased the likelihood of subsequent infection at the same location. By comparing abundance patterns of initial parasitism and subsequent parasitism of both gills we were able to determine the repeatability of specific sites of attachment.

RESULTS

There were no significant asymmetries in the copepod abundance of the left and right gills of all three trout populations ($p>0.09$, paired t-tests) (Figure 2). Therefore, site of attachment is randomly selected by the copepod. We examined the temporal variance in copepod infection amongst the two populations and discovered that the <1yr. old trout populations were infected with copepods approximately 3 weeks into the study and the prevalence for the >2yr. old trout population gradually decreased with time. The <1yr. old trout populations eventually reached a prevalence of 80% and then stabilized (Figure 3). The possibility that the location of initial infection will beget further infection at the same location was supported in our analysis. Indeed, the initial pattern of parasitism is a strong indicator of future parasitism, but only for that specific gill. For example, the initial abundance and future abundance for the left gill are closely related even 50 days later (Figure 4). This pattern did not hold for the right gill (Figure 5).

We analyzed the possibility that host weight could influence copepod abundance and rate of infection by correcting the copepod abundance for fish weight and then comparing abundance changes over time. There was a general decrease overtime in copepod abundance when corrected for host weight (Figure 6).

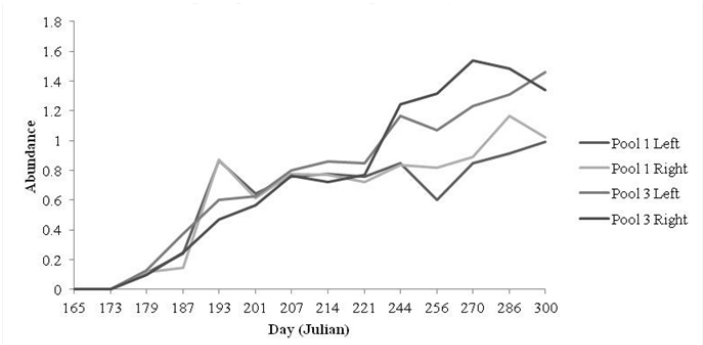

Figure 2. Left and right gill copepod abundance of <1yr. trout is not significantly different.

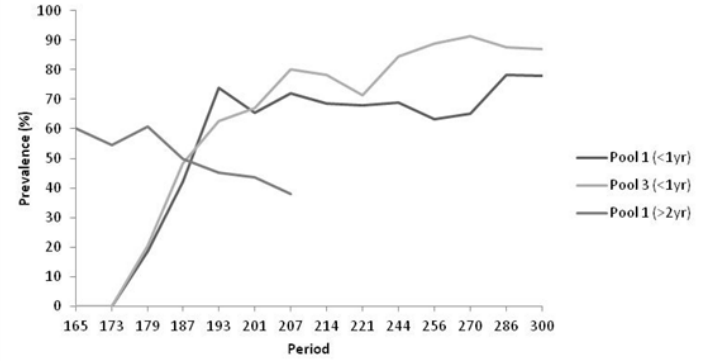

Figure 3. Summary of temporal variation in copepod infection.

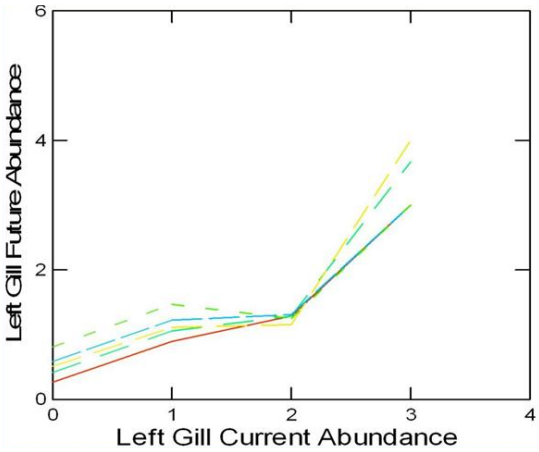

Figure 4. Influences of previous left gill parasitism (survey day 193) on subsequent pattern of parasitism of the left gill. Line indicate mean abundance on days 200, 207, 214, 221, and 244.

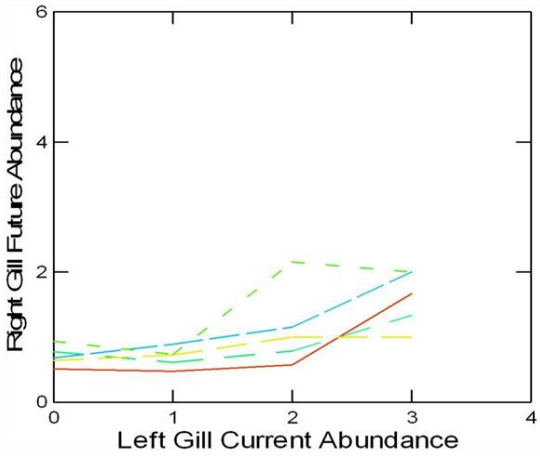

Figure 5. Influences of previous left gill parasitism (survey day 193) on subsequent pattern of parasitism of the right gill. Lines indicate mean abundance on days 200, 207, 214, 221 and 244.

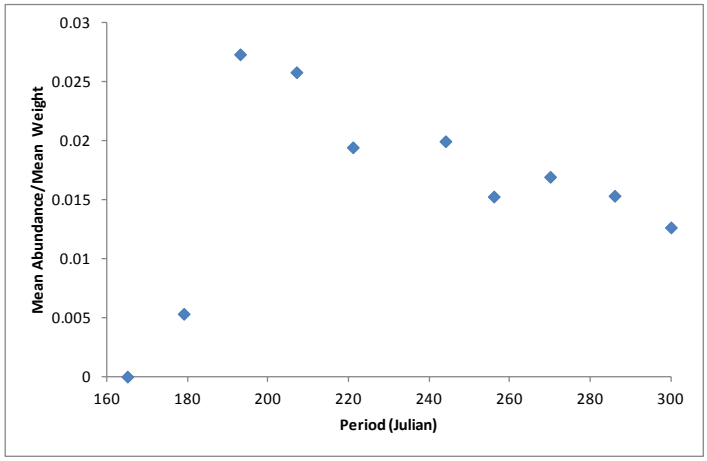

Figure 6. Mean abundance corrected for host weight over 140 day period.

DISCUSSION

The initial pattern of parasitism was not biased left or right, but subsequent parasitism was biased based on initial parasitism. This may be due to the damages incurred by the parasite to the host's tissue. *Salmincola californiensis* may also be able to overcome the host's defense mechanisms. By weakening the host at the specific site of attachment, the copepod that initially parasitized the trout would make it easier for other copepods to attach and survive. It was also positioned that the copepods are able to discern a more susceptible location that is ideal for attachment. Kabata and Cousens 1977 discuss the copepods' ability to freely move about the host before implantation occurs to find a mate, but it may be that the copepod is roaming about the host to determine the most susceptible location for attachment. There is also a possibility to believe that the copepods are simply attracted to one another and have evolved to be this way for easement of attachment, since choosing a site of attachment is a critical step in the life cycle of *Salmincola californiensis*. Finally, once an adult female releases her eggs while attached to the gill filament of the trout, some eggs remain tucked in the trout's branchial cavity due to the protruding operculum that surrounds the gill filaments. This is particularly plausible if water flow is impeded by the opercula such that infected fish are found to be continuously infected.

Size of the host is important when analyzing abundance; it is interesting that host weight seems to have a strong predictive potential. Weight may be a greater factor in the level of copepod abundance due to the host consuming more food and therefore being healthier and less susceptible to *Salmincola californiensis*. However additional morphometric and health-related factors could also correlate with abundance. To ensure our understanding of the parasite-host interaction of these species, further research is needed in regard to host size and physiology.

ACKNOWLEDGEMENTS

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Featured Scholar

MARY CLARK, EdD
Director Academic Advising
Housatonic Community College

I took my first college class when I was 29 years old. I was a single parent, a factory worker, and the first person in my family to go to college. My only goal was to earn a Bachelor's degree. Earning a doctorate was not on my radar, but all that changed when I came to MU and I discovered the McNair Program. I will admit I was intrigued mostly because of the stipend. I needed the money. I also knew getting into the program was a long shot. My GPA was satisfactory, but not stellar. However, in this particular year additional funding allowed 32 participants. I was one of them.

I began my work as a MU McNair Scholar in 1994. For the next year I worked as hard as I have ever worked in my life up until that point. I was fortunate that Dr. Helen Neville agreed to mentor me. She taught me how to conduct research, about data collection and the finer points of statistics. When we were done, I did a poster presentation in Chicago, went to a women's conference in Indiana and had my research published in the *MU McNair Journal,* only one of five selected. I graduated with my Bachelor's in Psychology in 1995 with honors and became a member of Psi Chi, the National Psychology Honors Society. The McNair Program helped prepare me for the GRE, waived my application fees for the twelve universities I applied to and paid for campus visits to potential universities. By the fall semester 1995, I began my Master's Program at New Mexico State University in Las Cruces. I completed my Master's Degree in Counseling and Guidance in 1997 and was poised to enter into the Ph.D. program when life happened. It would be a decade before I resumed my doctoral pursuits.

Steadfast as my life coach and mentor, former MU McNair Scholars Program Director Dr. Vicky Curby, stood by me, reminding me of my promise to earn my doctorate. When I was accepted into the University of Bridgeport's Doctoral program, one of my first tasks was to return to Missouri to give Dr. Curby the good news in person. Words cannot express the happiness I felt when I saw her again for the first time. I look at the picture taken of the two of us together on that day as a reminder of what it means to be a mentor and to make a promise. Earning my Doctorate in Educational Leadership in 2011, twenty-two years since I took my first college class, I also learned what it meant to keep one.

Education has been one of those endeavors in my life that I take without regret. I walk its path with confidence now but I often have to pinch myself as I can hardly believe sometimes that it was me who did this. As I remember my beginnings recalling my past and appreciating my present, I can only sit in awe of what has transpired. I don't think I am exaggerating when I say that it is a miracle that any first generation college student graduates at all. The odds are so great against us. Without programs like McNair there would be little hope for many of us. I personally sat on the cusp of failure, on its precipice with my feet dangling off the edge of it and yet I did not slip into the abyss. I may have wondered off the path occasionally, but I was never truly lost because deep within my soul I knew what I had to do because I knew I was not alone. Because of the McNair Program I was able to find the support I needed when I needed and that is what has made all the difference. If we are to be successful, we must believe in ourselves, but as Dr. Ronald E. McNair so aptly stated, "Before you can make a dream come true, you must first have one." Little did I know when I entered the doors of MU, my life would change. Little did I know when I applied to the McNair Program, I'd earn more than a stipend. I made a promise to the McNair Program and to Dr. Curby that I would earn my doctorate and when I crossed that stage in 2011, I fulfilled my promise to everyone especially to myself.

Featured Scholar

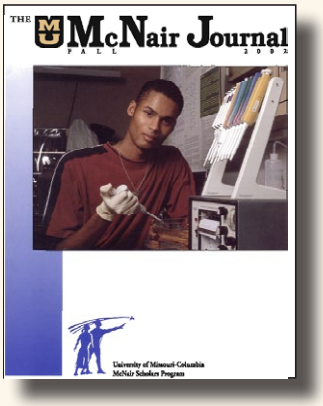
ALIAS SMITH, PhD
Assistant Professor, Biotechnology/Biology
Northern Virigina Community College

When I began my undergraduate education at Mizzou I did not have a clear career path in mind. Since I enjoyed science, I thought seriously about working towards medical school and becoming a physician, because that is what I thought was expected of Biochemistry majors. As I began to really dive into my upper division courses I was introduced to the idea of research. My professors stressed that there were a lot of questions out there to be answered, and, just as important, there are questions that have yet to be asked. They even made the point that there are education and career paths that are research centered. I was quickly sold on pursuing a career in research but I had little idea on how to make it happen, beyond the fact that it involved graduate school. My participation in the McNair program gave me the preparedness and confidence to apply for and meet the challenges of graduate school.

Through the support of the McNair program and my mentor, Dr. Lisa Sattenspiel, I discovered that I could devise a research project, collect data, and create mathematical models that can be used to ask specific questions. Furthermore, the experiences of conveying my findings in both poster and oral presentations, as well as in article form, have been invaluable. When I graduated from Mizzou I entered a graduate program in Microbiology and Molecular Genetics at the University of California, Los Angeles (UCLA). At UCLA I was quickly challenged to present research articles in seminar classes and propose research questions in the lab I joined. The skills I learned as a McNair Scholar helped me succeed in this program and I earned my PhD in the Spring of 2009.

During my graduate training my wife, who was also a graduate student, and I decided to start a family. We had two sons while completing our doctorates at UCLA. Maintaining a work-life balance was difficult, but I was able to employ several very helpful concepts presented at a McNair sponsored panel that discussed work-life balance. Through my participation in the McNair program I not only acquired valuable skills that helped me earn my graduate degree, but I also gained guidance on how to be successful and healthy in multiple aspects of life in and beyond graduate school.

After UCLA I went to the University of California, San Diego were I spent two years as a Postdoctoral Fellow and furthered my training in microbiology and molecular genetics. In addition to research I became an adjunct professor at National University and taught General Biology courses in the evenings. It was a challenge to balance research and teaching with family life, but overall it was a positive experience and I fell in love with teaching. At the completion of my Postdoctoral Fellowship I moved to the Washington DC and successfully competed for a Biotechnology/Biology Assistant Professor position at the Northern Virginia Community College (NOVA). I am currently involved in teaching, mentoring, and writing grants to further develop the Biotechnology program at NOVA, all of which make use of skills I began to develop as a McNair Scholar.



Alias Smith from the cover of the *Fall 2002 MU McNair Journal.*

2011-2012 Research Topics

Scholar	Major	Title	Mentor
Bianca Aaron	Journalism & Psychology	Effects of Language Brokering and Gender on Latina Adolescents	Alejandro Morales
Brittany Bennett	Business Marketing	Brand Loyalty: A Matter of the Maximizing Trait and Product Category Involvement?	S. Ratneshwar
Brittani Bungart	Biological Engineering	Nanoparticle-emitted Light on Alzheimer's Disease-Related Pathways	James C. Lee
Darnell Cage	Mechanical & Aerospace Engineering	Improved Methods of Cryopreservation of Biological Specimen Using Geometrically Modified Vials	Gary L. Solbbrekken
Marc Canellas	Mechanical & Aerospace Engineering	Development of Planetocentric Reference Frames to Model the Flyby Anomaly	Sergei Kopeikin
Timothy Cunningham	Business Management	Testing Intercultural Consultant's Perceptions of National Cultural Differences Between China and the United States	Douglas Moesel
Megan Dowdle	Biochemistry	Ribosome Interactivation Modulated by Hibernation Promoting Factor and Ribosome Moulation Factor	Peter Cornish
Jeneé Duncan	Human Development & Family Studies	African-American College Students in Long Distance Dating Relationships: A Mixed Methods Study	David Schramm
Carmen Harjoe	Fisheries & Wildlife	Effects of Previous Experience on Aggressive Behavior in Male Gray Treefrogs, *Hyla versicolor*	H. Carl Gerhardt
Bethany Henry	History & Anthropology	National Parks and the Preservation of Indigenous Heritage in the United States and Canada: Comparing Web Images and Policy	Craig Palmer
Damir Kolasinac	Biological Engineering	Development of a Sensor for the Detection of Explosive Compounds Utilizing Fluroescently Labeled Molecularly Imprinted Polymer Conjugates on Electrospun Scaffolds	Sheila Grant
Carolyn Lacey	Biochemistry	Sorghum Somatic Embryogenic Callus Induction and Plantlet Regeneration	William Folk
Gerald Mitchell	Psychology	Positive Affect and Global Focus: The Moderating Effects of Intuition	Laura A. King
Danielle Mocker	Fisheries & Wildlife	Parasitism of Hatchery Rainbow Trout by *Salmincola Californiensis*: Attachment Sites, Duration of Infection, and the Ontogeny of Parasite Aggregation	Matthew E. Gompper
Akia Parks	Biological Engineering	Photoacoustice Detection of Escherichia coli Bacteria Cells for Screening of Septicemia	John A. Viator
Rosalyn Reese	Biochemistry	Refining Methods for Detecting Heteroplasmic Mitochondrial DNA Mutations	William Folk
Antaniece Sills	Human Devlopment & Family Studies	The Influence of Parenting Styles on Collegiate Students Academic Success	Kalea Benner
Caitlin Vore	Environmental Science & Forestry	Sniffing Danger: Variation in Volatile Profiles of Insect herbivory on *Arabidopsis thaliana*	Heidi Appel

2011-2012 McNair Scholars

Back row: Jeremy Bloss (Student Services Advisor), LaShonda Carter-Boone (Research Seminar Instructor), Timothy Cunningham, Damir Kolasinac

Second Row: NaTashua Davis (Director), Caitlin Vore, Carmen Harjoe, Marc Canellas, Rosalyn Reese, Darnell Cage, Gerald Mitchell, Darlene Dixon (Program Assistant)

First Row: Bianca Aaron, Brittani Bungart, Carolyn Lacey, Akia Parks, Brittany Bennett, Antanice Sills, Megan Dowdle, Jeneé Duncan

Not Pictured: Bethany Henry, Danielle Mocker

McNair Scholars Program

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