

# DISCUSSIONS

CASE WESTERN RESERVE UNIVERSITY UNDERGRADUATE RESEARCH JOURNAL

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## FEATURING:

Development of a Micro-integration Technique  
for the Fabrication of a Cell-Fiber Scaffold  
Complex in Heart Valve Tissue Engineering  
*Qi Jia*

Four Lines to Immortality: Dido's Renaissance  
Through Josquin des Prez  
*Dana M. Plank*

The Role of Innate Receptor TLR2 in Neutrophil  
Recruitment in Oropharyngeal Candidiasis (OPC)  
*Saleh Al-Kharsa*

## Additionally:

*Maria Theodorou  
& Ryan Miller*

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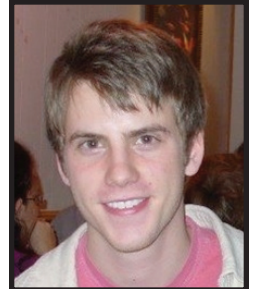
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# LETTER FROM THE EDITORS

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Dear Reader,

It is with great joy and pride we present to you the second issue of the fifth volume of *Discussions*, CWRU's interdisciplinary peer-reviewed undergraduate research journal. Having been here since the starting phase of the journal, it is such a wonderful feeling to see how the journal has grown and been accepted by the student body on campus. We would like to take this opportunity to thank the entire CWRU community for their positive reception of *Discussions* over the past few years.

With this issue, we bring you five exceptional papers with topics encompassing immunology, biomedical engineering, ecology, and for the first time, music. Our authors have accomplished a great deal in their time at CWRU and we hope that this issue grants you a glimpse of their achievements. We also invite the campus to our annual author reception on Thursday, April 23<sup>rd</sup>, 2009 to honor these and last issue's authors. The event will take place from 11:30am to 1:00pm in the 1914 Lounge in Thwing Center.

*Discussions* would never have made it off the ground, let alone stood on its own feet, if it hadn't been for the unwavering support and dedication of our adviser and mentor, Dr. Shelia Pedigo, Director of the SOURCE Office. She has been with us every step of the way, attending meetings, offering advice, and sharing a genuine concern for the journal and its members. Our Web Adviser, Bethany Pope, has aided us in maintaining a website while we work on obtaining a separate site. Without her help we would not be as easily accessible as we are. Thank you Dr. Pedigo and Ms. Pope for your continued guidance and support.

We hope that you will enjoy reading the articles in this issue and appreciate the research undergraduates on our campus perform everyday.

Sincerely,

Aditi Sinha and Sean Yeldell

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# DEVELOPMENT OF A MICRO-INTEGRATION TECHNIQUE FOR THE FABRICATION OF A CELL-FIBER SCAFFOLD COMPLEX IN HEART VALVE TISSUE ENGINEERING

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-Qi Jia-

Qi Jia is a third year student at Case Western Reserve University pursuing a degree in Chemistry and minoring in Chinese and Biology. After graduation, she plans on attend medical school. Qi is an active member in Alpha Chi Sigma, American Medical Student Association, Golden Key International Honour Society, and Koinonia Christian Fellowship. Currently, she is doing research on both heart valve tissue engineering in the Macromolecular Science & engineering Department and on biochemical/organic synthesis in the Chemistry Department. In her spare time, she enjoys painting and ice-skating.

### -Acknowledgments-

The effective collaboration and cooperation among all members of our interdisciplinary team have made this work successful. Specifically, I would like to thank Dr. Gary Wnek for his guidance and expertise in polymer science, electro-spinning and biomaterials, as well as providing access to polymer processing facilities at Case Western Reserve University Department of Macromolecular Science and Engineering. I would also like to thank Dr. Yakov Elgudin and Dr. Peter Havernik from the Case Western Reserve University School of Medicine for providing considerable expertise in heart valve tissue engineering, tissue culture, delivery systems, cell biology and histology. I must also thank Meghan Smith for her patience, supervision and invaluable assistance in carrying out lab procedures and techniques. Lastly, I want to thank everyone else for their support in making this research project possible.

### ABSTRACT

All clinically available heart valve prostheses, such as mechanical and bio-prosthetic valves, are intrinsically flawed in that they are unable to grow and become a fully functional part of the native tissue. In response to this critical need for a better heart valve substitute, we aim to develop a multifunctional scaffold that ideally mimics a native heart valve in mechanical properties, serves as a growth factor and cytokine delivery vehicle, and supports cell attachment and proliferation through tissue engineering. An innovative technique known as micro-integration, involving simultaneous electro-spraying of cells and electro-spinning of fiber matrix, is designed and optimized for uniform and selective positioning of cells within a scaffold. The effectiveness of this process, as well as cell viability, proliferation and location in scaffold are then assessed using live/dead cell assay and fluorescence and confocal microscopies. Preliminary work involves electrostatic spraying of fluorescent NIH 3T3 fibroblasts, endothelial progenitor cells and, eventually, mesenchymal stem cell into a polymer scaffold made from solutions of biodegradable and biocompatible polymers such as poly-( $\epsilon$ -caprolactone) in chloroform. Optical and scanning microscopy images of a seeded scaffold fixed in paraformaldehyde indicate improved cell growth and adhesion on fibrous surfaces with small pores in comparison to a smooth film of the same polymer. Fluorescent imaging shows uniform cell distribution throughout the scaffold. Unfixed portions of the scaffold cultured for subsequent assessment illustrate approximately 80% cell viability and growth via cell counting and live/dead stain. Future work will focus on improving the scaffold through controlled delivery of key growth factors and ensuring adequate nutrient and waste transport through controlled porosity. The expected outcome of this work is a state-of-the-art methodology for tissue engineering scaffold optimization which will lay a strong foundation for achieving the long term goal of developing a fully functional regenerated heart valve.

### INTRODUCTION

The study of heart valve disease is an emerging area of research made possible by the design of in vitro and in vivo cellular experimental involved in development of synthetic valves. Heart valve disease is a health care concern that draws much attention in society as it affects a significant portion of the international population. Every year, about 5 million Americans learn

they have heart valve disease (Nkomo et al., 2006). The common treatment of end stage valvular disease is valve replacement, followed by prosthetic valve implantation. Currently, the two types of prosthetic valves available—mechanical and synthetic valves—are both flawed. A principal limitation of the use of mechanical prosthetic valves is the need for a life-long anticoagulation to treat thromboembolism. While synthetic valves do not require continuous anticoagulant use, they are only durable for 10 to 15 years due to matrix calcification, which requires frequent reoperation. The operation-associated complications increase patients' morbidity, mortality and financial burdens. Furthermore, the fact that all clinically available heart valve prostheses cannot grow and function fully as part of the native tissue is a particular concern in pediatric patients who are still growing (Fong, Shin'oka and Lopez-Soler, 2006). Clearly, there is an urgent need for an improved heart valve substitute, which can be accomplished through the use of tissue engineering and regeneration.

The advantage of investigating synthetic scaffolds is that they allow for controlled development of valve mechanical properties and varied matrix structure obtained from different types of polymers. Tissue engineered scaffolds act as a template for complex heart valve construct re-growth. Ideally, scaffolds should facilitate cell attachment and proliferation, provide an advantageous biochemical environment, and enable adequate nutrient and waste transport. Types of polymers available for fabricating scaffolds include poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid), and poly(caprolactone). More commonly, a combination of these biocompatible and biodegradable materials is used. However, the rigid nature of the polyesters prevents the scaffolds from having similar mechanical properties and an architecture as complexly layered as the native leaflet. Many synthetic matrices also limit cell adhesion and scaffold infiltration (Mendelson and Schoen, 2006). Thus, scaffold development for heart valve tissue engineering needs improvements in both mechanical and cellular components. The long term goal of this work focuses on developing a multifunctional scaffold that: 1) possesses mechanical properties that meet the tissue engineering application requirements, 2) serves as a controlled multiple growth factor and cytokine delivery vehicle, 3) is capable of supporting cell attachment and proliferation and 4) influences progenitor cell differentiation through both physical and biochemical stimuli. This will be accomplished through a multidisciplinary approach

involving the following: mathematical modeling and experimental design of biopolymer scaffolds with targeted mechanical properties and degradation rate, development of a controlled growth factor delivery model, and an innovative electro-spraying-electro-spinning methodology (known as micro-integration) of cell incorporation into the biologically active scaffold. The immediate aim of the work presented in this paper is to enable selective distribution of endothelial cells and fibroblasts throughout the scaffold matrix via the micro-integration method, and to promote and assess cell viability and proliferation within the scaffold using fluorescence and optical microscopy. Histological staining and protein expression are used to evaluate the biological impact of the scaffolds with and without incorporated growth factor release.

The electro-spinning technique is capable of producing micro- or nanofibrous scaffolds with similar morphological characteristics to natural extracellular matrices (Bowlin et al., 2002). It is a means of polymer processing making use of electric forces to form small diameter polymer fibers and non-woven mats of those fibers. In practice, the polymer solution loaded in a syringe is charged by a strong electrical potential to the metal needle, which is in the presence of a grounded target located on a rotating metal a few centimeters away. The application of the potential results in a charged jet of the polymer solution being released from the tip of the needle in the formation of a Taylor cone. The jet travels through the air to a grounded collecting drum, leaving a solid polymer fiber. As fibers start to collect with time, a mat is formed. The jet diameter is usually a small fraction of that of the needle, and the resulting fiber diameters and pore sizes can vary depending upon the polymer flow rate and the distance between the tip of the needle and the grounded target. In the electro-spraying process, drops of cells dispersed in cell media shoot out from the tip of another needle toward the same drum upon being charged by the electrical potential. Thus, the electro-sprayed cells are entrapped within the selected areas of the fibrous matrix in the formation of the cell-fiber construct.

## METHODS

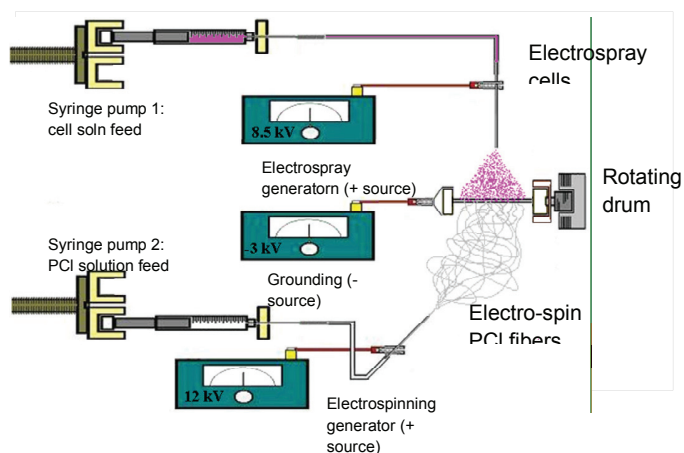
### *Cell Selection and Culture*

Preliminary studies involved seeding GFP+ (green fluorescent) 3T3 fibroblasts into poly-(caprolactone) (PCL) scaffolds. GFP+ fibroblasts were chosen for the convenience of fluorescence spotting in optical imaging. Later

experiments involved the use of endothelial progenitor cell (EPC) derived from umbilical cord blood, CD133+. This cell type is closest in replicating the natural cellular component found in the native leaflet tissue. To obtain these cells, mononuclear stem cells (MNSCs) were first isolated from samples of cord blood by conventional centrifuge methods through a Ficoll-density gradient. Whole blood samples were placed on top of Ficoll and then were centrifuged at 1,500 rpm for 30 minutes with the break open. Before isolation and seven days of culturing, recovered MNSCs were sucked and removed from the tube and placed into a new centrifuging tube containing phosphate-buffered saline (PBS). The culture media used was Medium-199, supplemented with 20% fetal bovine serum (FBS) and antibiotics. During this period, cell maturing and proliferation was induced. Flow cytometry was then used to identify the CD133+. In preparation for the micro-integration process, the cells were detached using 0.05% trypsin from the flasks, where they were suspended in Medium-199 and DMEM, respectively, with 10% FBS. Then, cells were counted and placed in a sterile 5 mL syringe.

#### Micro-integration Process

PCI served as a starting point for our material investigation. A 12 wt% solution of PCI in chloroform was loaded in a 5 mL syringe with a blunt tipped 18 gauge stainless steel needle and horizontally positioned with respect to a sterilized, rotating metal drum with a surface area of 12.5 cm<sup>2</sup>. A second syringe containing approximately 4 x 10<sup>6</sup> fibroblasts in 0.6 mL culture media was positioned vertically above the collecting drum. A positive 15 kV voltage was applied to both syringes. In order to enhance fiber collection via opposite polarity, a negative 3kV voltage was applied to the drum instead of grounded. The electro-spinning flow rate was set at 1 mL/hr with the syringe positioned 0.8cm away from the target. The flow rate for electrostatic spraying process was 0.3mL/hr, and the syringe containing cells was placed at 0.4cm away from the target. Then, electro-spinning of the polymer and electro-spraying of the cells were carried out simultaneously, as shown in Figure 1. The scaffold was allowed to build up to approximately 100 μm in thickness and then removed from the drum. A portion of the scaffold was removed, placed in growth media and kept in an incubator (37°C and 5% CO<sub>2</sub>). The remaining scaffold was fixed in paraformaldehyde for imaging using optical and fluorescent microscopy.



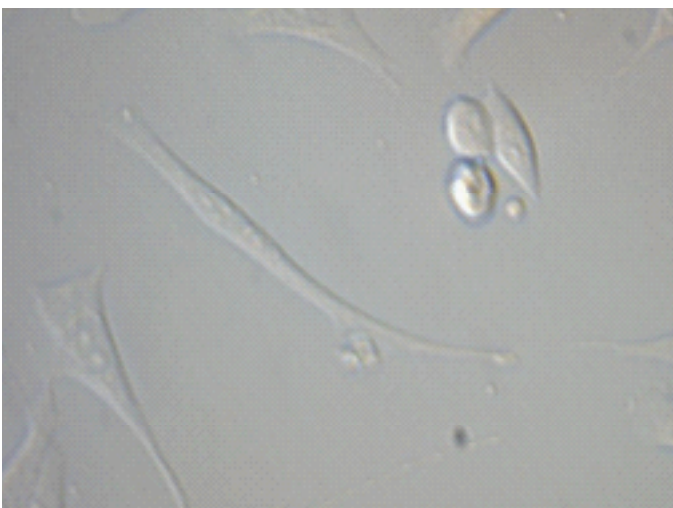
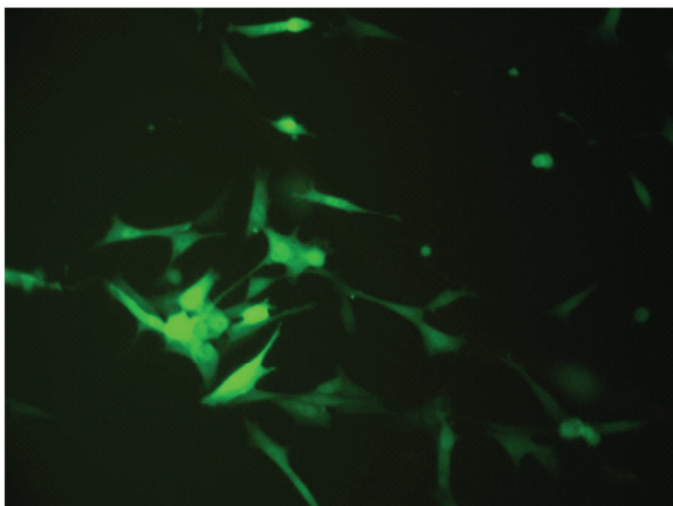
**Figure 1:** Experimental design of micro-integration set-up involving simultaneous electro-spraying of cells and electro-spinning polymer.

Later experiments focused on developing scaffolds consisting of multiple cell types and polymers. This was accomplished using a sequential approach to construct and form various layers. Outer layers consisting of collagen electrospun from hexafluoroisopropanol (HFIP) were combined with electro-sprayed EPCs. These collagen layers were sandwiched around an inner layer of electrospun PCI containing electro-sprayed mesenchymal stem cells. This allowed for the development of a scaffold consisting of two localized cell populations in a layered multi-component polymeric scaffold. Once constructed, the mats were kept in culture conditions with DMEM media with 2% fetal bovine serum. Electrospun mats were then characterized via scanning electron microscopy for fiber diameters and porosity. Cell distribution within the scaffold was identified through hematoxylin and eosin staining and visualized with light microscopy. Cell viability was also assessed using the dead/alive assay.

## RESULTS

Initial studies were carried out using a single polymer/cell types construct: (PCI)/(GFP+ 3T3 Fibroblasts). Live and functioning cells spread themselves out on a surface in the attaching process. Microscopic images of the cells in the electrospun PCI sample taken at 1, 3, 7, 10 and 14 days after initial seeding showed increasing cell diameter, which suggests excellent cell attachment and growth. Visualization of attaching cells is shown in Figure 2 (right).

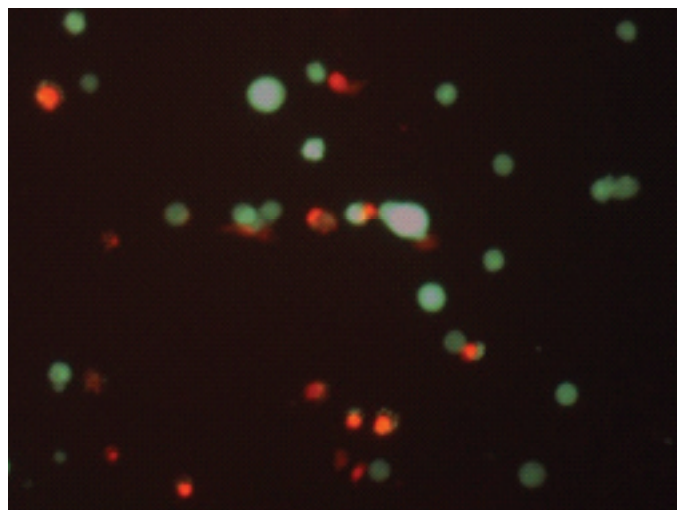
A live/dead fluorescent stain performed on these cells confirmed cell viability and proliferation as illus-



**Figure 2:** fluorescence and light microscopy of alive and well-attaching GFP+ 3T3 fibroblasts before electrostatic seeding

trated in Figure 3. The green fluorescence indicates living cells, while the red fluorescence shows the amount of dead cells present in the sample. The image shown in Figure 2 illustrates the presence of a majority of green fluorescently stained cells. This result provided further evidence for cell viability and proliferation.

To determine statistically the number of live cells in the sample after the initial 24 hour period, dead cells were washed away with PBS, and the remaining alive ones were counted manually. Cell counts data were taken from 10 equally sized areas within the scaffold. Subsequent calculation of live and dead cell averages and deviations showed approximately 80% cell viability in comparison to the total number of cells counted before the electro-spinning/spraying process. In addition, even number of live



**Figure 3:** Image of 3T3 fibroblast/PCI sample stained with the alive/dead fluorescence assay. Green indicates live cells and red indicates dead cell.

and dead cell counts determined at different thicknesses and widths of the scaffold also demonstrated uniform cell densities and cell distribution via the micro-integration process. Figure 4 (next page) presents visual images of homogenous cells distributed throughout the matrix taken by light and fluorescence microscopy.

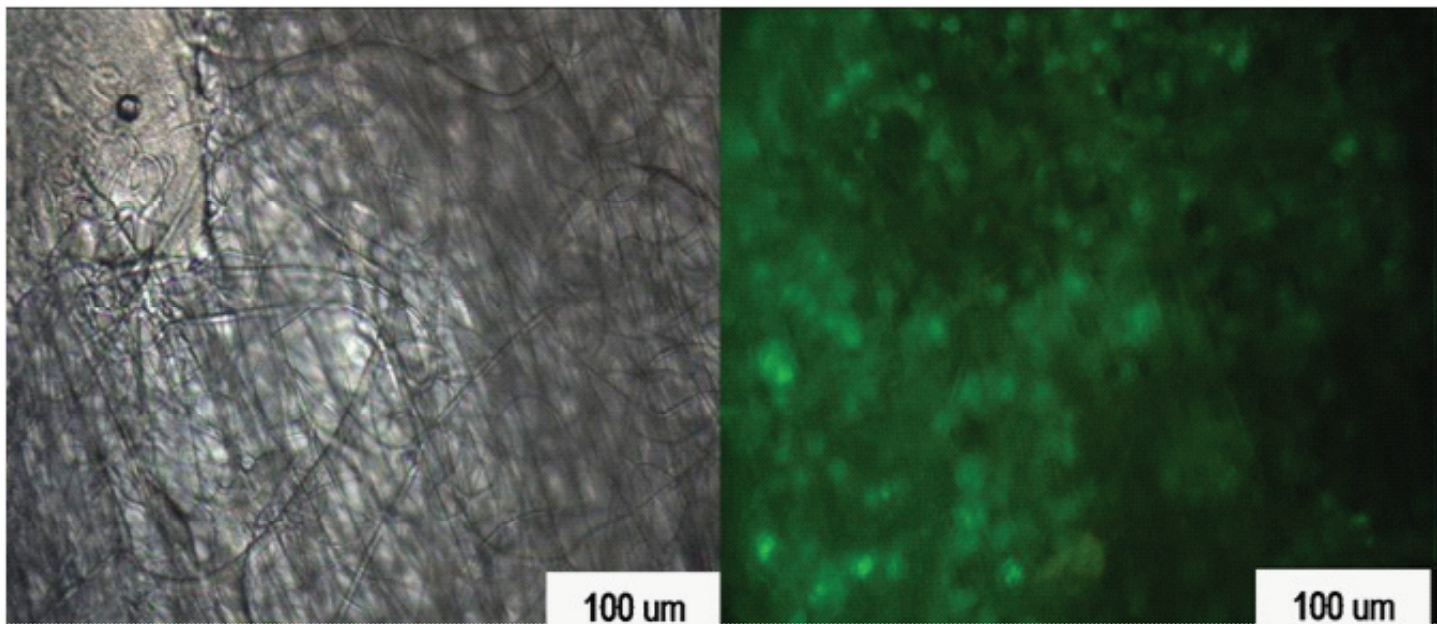
Furthermore, optical and scanning microscopy images of a portion of seeded scaffold fixed in paraformaldehyde indicated improved cell growth and adhesion on fibrous surfaces with small pores, in comparison to a smooth film of the same polymer. These data were obtained by comparing cell growth profiles on a mat of electrospun PCI versus a PCI cast film.

Subsequent experiments involving the endothelial progenitor cells and the fibroblasts seeded in layers of electrospun collagen and PCI also showed good cell viability and uniform cellular distribution. Histological sectioning and staining of the samples are still in progress.

## DISCUSSION

In these experiments, an innovative approach involving simultaneous electrospinning of polymer and electrospraying of cells was successfully developed and optimized in seeding of the GFP+ 3T3 fibroblasts and the progenitor cells in the electrospun fibrous scaffolds. Cell viability and proliferation was maintained both through the electrospinning process and subsequent culturing of the constructs. Cells were also found to be uniformly distributed throughout the cross section of the scaffold samples. The optimiza-





**Figure 4:** Light and fluorescence microscopy of electrospun poly(caprolactone) scaffolds seeded with green fluorescent protein expressing 3T3 fibroblasts via electrostatic spraying, showing the uniform distribution of cells throughout the matrix.

tion of the electrospin/spray process was achieved through varying the electro-spinning processing parameters, such as the flow rate of the polymer solution and the distance between the syringe and the drum. Varied fiber diameter and pore sizes were also obtained by controlling these parameters. During the micro-integration process, we overcame the difficulty in keeping cells alive during the two hour electrospray process by constantly spraying media onto the developing cell/fiber construct. We also coated the fibers with laminin to promote better cell adhesion to the synthetic components of the scaffold. Upon fabrication of the cell-seeded scaffolds, the unfixed sample portion cultured in cell media showed substantial viability and differentiation over time. However, we believe that we can induce further cell proliferation by adding growth factors.

Data on fixed samples processed for histological examination with hematoxylin and eosin staining to identify cell location within the scaffold upon initial seeding will be obtained in the next set of experiments. Also, maturation of seeded cells from progenitor forms will be assessed based on their genes and protein products by multiplex protein analysis and real-time PCR analysis. The extent of cell differentiation from precursor to mature phenotype induced by the scaffold with or without growth factor delivery must be determined in order to demonstrate valve-like histological cell layering and cell type matched matrix secretion.

## CONCLUSION

The expected outcome of these experiments showed that 1) cells survived the electrostatic seeding process and 2) cells adhered to the fibers and were viable and multiplying within the scaffold. Uniform distribution of cells within the scaffolds also indicated successful application of the micro-integration process. These preliminary investigations showed promising results in the development of both a multifunctional scaffold and a state-of-the-art methodology for tissue engineering scaffold optimization, which will lay a strong foundation for future work towards generating a fully functional heart valve substitute.

In future experiments, several other polymers will be considered for scaffolding, either on their own or as part of a multi-polymer scaffold. These polymers include, but are not limited to, synthetic biocompatible materials such as PLGA, PGA, poly-4-hydroxybutyrate, and several poly(urethanes). Natural polymers such as collagen, elastin, and fibrinogen will also be investigated for incorporation with the synthetic polymers in an attempt to better mimic the native cellular architecture and the mechanical properties of the native heart valve tissue.

The next stages of work will concentrate on producing a tri-layer scaffold with endothelial precursor layers on the top and bottom, sandwiching a center layer of fibroblast progenitor cells, and determining the biocompatibility and hemodynamic function of the engineered valve in a bio-reactor system, prior to valve implantation and testing in mice models.

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# PROXIDANT INJECTION CAUSES THE ONSET OF TYPE 2 DIABETES IN THE SPONTANEOUSLY HYPERTENSIVE OBESE (SHROB/KOL) RAT

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## -Maria Theodorou-

Maria is a fourth year student studying Spanish, Biology, and Chemistry at CWRU. She is active in the Office of Undergraduate Admissions, Greek Life, and is a volunteer at Rainbow Babies and Children's Hospital. Upon completion of her undergraduate studies, Maria plans to attend medical school. Maria enjoys traveling, downhill skiing, and Zumba.

## -Acknowledgments-

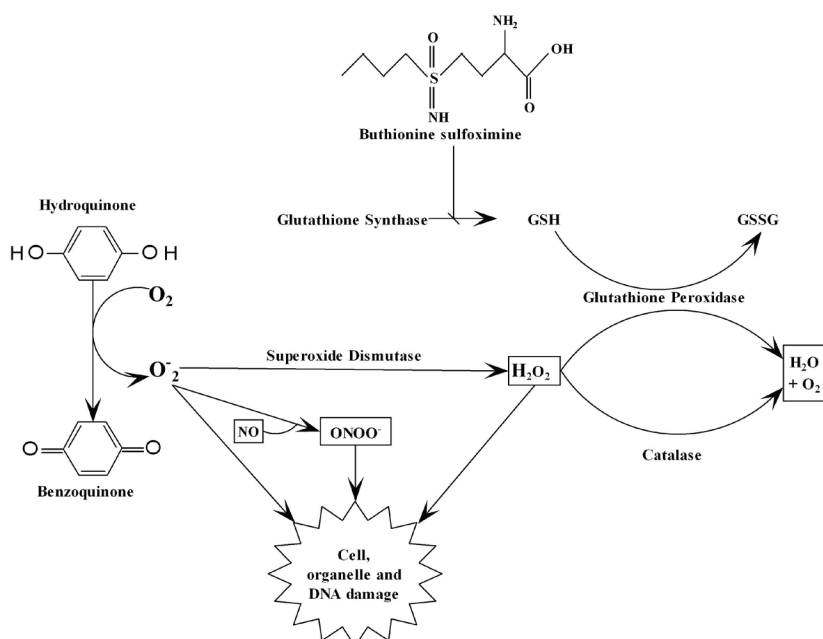
I would like to thank my advisor Dr. Paul Ernsberger and our co-investigator Dr. Richard Koletsky for their guidance and support of my research endeavors. Thank you to Rachel Koletsky, Neema Patel, Alex Moore, Matt Koletsky, Liza Escobedo, Sabrina Jackson, and Simone Edwards for their help in conducting this research. I would like to thank the Dietrich Diabetes Research Institute of the Diabetes Association of Greater Cleveland and the SOURCE Office of Case Western Reserve University for their funding, and CWRU's Department of Nutrition for serving as home base for my research over the past three years.

## ABSTRACT

The spontaneously hypertensive obese (SHROB/Kol) rat is a model of pre-diabetes characterized by normal fasting and high postprandial glucose and insulin resistance. Oxidative stress, through the damaging effects of oxygen radicals, may contribute to the onset of diabetes. Oxidative stress was induced in SHROB with a combination of a prooxidant agent, hydroquinone, and a glutathione synthase inhibitor, L-buthionine sulfoximine (both 50 mg/kg ip). Following prooxidant injections, plasma peroxide levels were increased within 1 h and returned to baseline within 24h. Diabetes, defined as basal blood glucose in excess of 126 mg/dL, appeared within two days of daily treatment. Morning blood glucose values averaged over 200 mg/dl in SHROB injected with pro-oxidant solution versus about 100 mg/dl in control SHROB injected with saline. Glucose fell to baseline within 3d after cessation of prooxidant treatment, but glucose tolerance remained significantly impaired. Food intake and body weight were not significantly affected by prooxidant treatment at any point. Oxidative stress was measured by assay of peroxides in plasma. The SHROB rat subjected to oxidative stress is a potential model to study the onset of Type 2 diabetes, and supports the hypothesis that oxidative stress may be a trigger for diabetes onset in susceptible individuals.

## INTRODUCTION

Approximately 15 million Americans suffer from diabetes today (Candib, 2007). This serious illness exists in two forms, type 1 and type 2. Currently 5% of diabetics suffer from type 1, while 95% of diabetics suffer from type 2. Type 1 diabetes results from the autoimmune-mediated destruction of insulin-producing beta cells, which causes an absolute insulin deficiency. Multiple factors contribute to type 2 diabetes, including the combined influence of genetic susceptibility and environmental factors such as nutrition, obesity, age, and physical inactivity. In the early stages of the development of diabetes, prior to the onset of high glucose levels, these contributing factors result in insulin resistance compensated by insulin production that is adequate or even excessive (Candib, 2007). The next stage in the progression to diabetes is impaired glucose tolerance (Benjamin, Valdez, Geiss, Rolka, & Narayan, 2003). Human metabolic syndrome is characterized by a group of metabolic risk factors, including abdominal obesity, dyslipidemia, hypertension, insu-



**Figure 1:** Experimental intervention in oxidative stress pathways. Rats were given hydroquinone, which yields superoxide when reduced to quinone. Additionally, the rats were given buthionine sulfoximine, a glutathione synthase inhibitor, which depletes the supply of this intracellular antioxidant. This reduces the breakdown of hydrogen peroxide and also interferes with other redox reactions in which glutathione participates. The combination of hydroquinone and buthionine sulfoximine leads to an imbalance of oxidative and antioxidant processes within cells, a condition known as oxidative stress.

lin resistance, and impaired glucose tolerance. Metabolic syndrome is thought to play a role in increasing the risk for type 2 diabetes.

Obesity contributes to all components of metabolic syndrome, especially in the presence of excess abdominal fat (Batsis, Nieto-Martinez, & Lopez-Jimenez, 2007). Increased weight, especially that caused by excess fat in the abdominal region, is associated with insulin resistance. For this reason, obesity is strongly associated with type 2 diabetes. However, this relationship is not absolute, as there are obese individuals who do not develop type 2 diabetes, as well as non-obese individuals who develop the disease (Karter et al., 2005). It is unclear why this is the case, leading to the question of which components in the body trigger diabetes in susceptible individuals.

A proposed mechanism for the insulin resistance implicated in metabolic syndrome, and subsequently in type 2 diabetes, is through oxidative stress. Oxidative stress is characterized by an imbalance between the production of reactive oxygen species and a biological system's ability to detoxify reactive intermediates or easily

repair the damage caused by these intermediates (Haidara, Yassin, Rateb, Ammar, & Zorkani, 2006). The natural cell environment of all living systems is reducing and this state is, for the most part, maintained through the action of certain enzymes which constantly provide a source of metabolic energy. The production of reactive oxygen species (ROS) disturbs this normal redox state through the production of peroxides and free radicals, substances which have been shown to have a damaging effect on almost all components of the cell and which may ultimately lead to cell death. This process is described in Figure 1.

Superoxide is a major reactive oxygen species in cells. As indicated in Figure 1, it directly damages cells, and reacts with nitric oxide to form an additionally damaging peroxynitrite radical. Superoxide is rapidly acted upon by superoxide dismutase to produce hydrogen peroxide, which is also detrimental to cells. Hydrogen peroxide is subsequently broken down by two pathways. First, the enzyme catalase works directly on hydrogen peroxide to produce water and molecular oxygen. Additionally, glutathione peroxidase breaks down

hydrogen peroxide to oxidized glutathione (GSSG, with a disulfide bridge) with the help of the reduced form of glutathione (GSH, with a free thiol group) (Figure 1). The chemical stimulation of these pathways through the injection of hydroquinone and buthionine sulfoximine leads to oxidative stress, the imbalance of oxidative and antioxidant processes within cells.

Oxidative stress has been implicated in the onset and progression of many diseases, including cancer, atherosclerosis, Parkinson's disease, Alzheimer's disease, and diabetes mellitus. In regards to diabetes, oxidative stress may contribute to pre-diabetic conditions such as insulin resistance and glucose intolerance, to diabetes itself, and to its complications such as retinopathy, nephropathy, neuropathy, stroke, and myocardial infarction (Keaney, Jr. et al., 2003). In this study, the effect of oxidative stress on blood glucose levels and the onset of diabetes was studied.

Antioxidants form part of the oxidative stress pathway and aid in the degradation of hydrogen peroxide to oxygen and water. Endogenous antioxidants include superoxide dismutase (SOD), glutathione, and catalase. An-



SHR 294g  
Spontaneously  
Hypertensive



SHROB 530g  
Obese Spontaneously  
Hypertensive

**Figure 2:** Pictures of typical SHROB and SHR female rats at 12 weeks of age

tioxidants can also be obtained exogenously from the diet, and are found in foods such as tomatoes, carrots, berries, garlic, and soy. Garlic's active principle, allicin, is thought to contribute significantly to the compound's antioxidant properties (Ernsberger, Johnson, Rosenthal, Mirelman, & Koletsky, 2007).

Antioxidants are thought to decrease components of metabolic syndrome, including blood pressure, fat content of the bloodstream, and insulin resistance. Additionally, measuring levels of antioxidants present in the cell is one way to measure the degree of oxidative stress, as an increased presence of antioxidants signifies an increased response to prooxidant stress. It is hypothesized that the mechanism of the onset of diabetes may be caused by oxidative stress in combination with obesity and diet, especially a diet lacking antioxidants.

The SHROB rats used in this experiment exhibited metabolic syndrome (Figure 2). Metabolic syndrome is caused by genetic abnormalities and can be influenced by diet, physical activity, and medicines (Koletsky, Velliquette, & Ernsberger, 2007; Koletsky, Velliquette, & Ernsberger, 2003). This syndrome is characterized by abdominal obesity, dyslipidemia, hypertension, insulin resistance, and impaired glucose tolerance. These symptoms are all associated with, and often lead to, diabetes in humans; however, untreated SHROB rats remain in a prediabetic state. The obesity exhibited by this model results from a single point mutation leading to a premature stop codon in the leptin receptor, preventing translation of functional

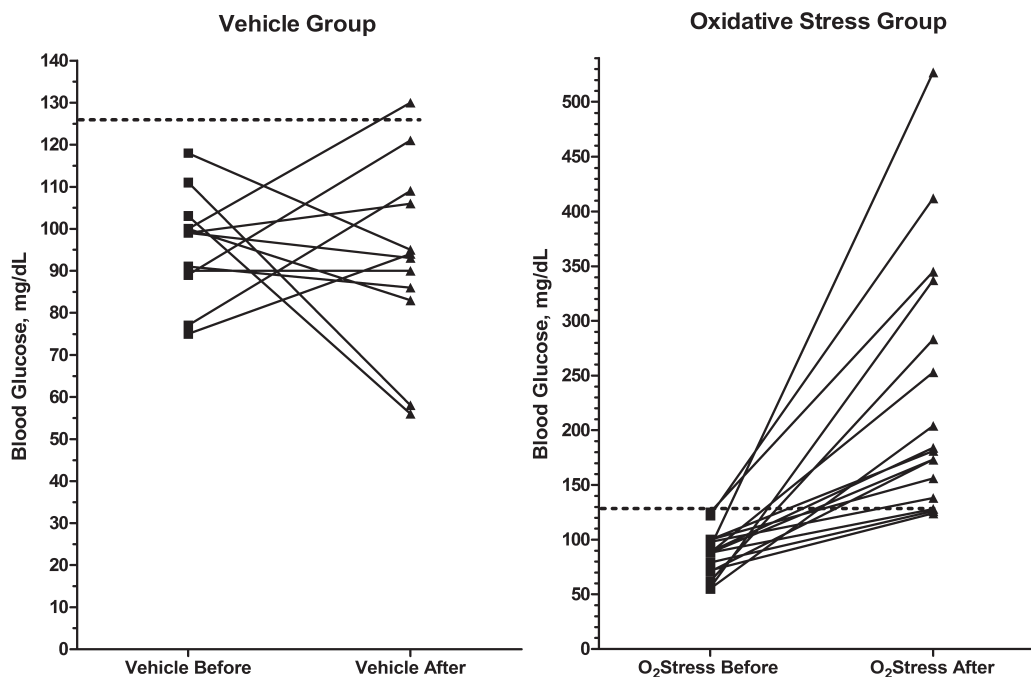
leptin receptors. Without these receptors, the effects of leptin on satiety are disrupted, and cause eating behaviors which lead to obesity. Other genetic mutations are responsible for the model's hypertension and dyslipidemia. However, these genes have not yet been fully identified. In combination, these genetic abnormalities form metabolic syndrome. Recently, a three-fold increase in plasma and organ lipid peroxides has been noted in SHROB relative to lean SHR littermates (Ernsberger, Theodorou, Edwards, & Koletsky, 2008). As such, we hypothesized that further increasing the elevated levels of oxidative stress in SHROB will be sufficient to trigger diabetes in this model.

## METHODS

SHROB rats were obtained from Charles River at 10 weeks of age. Body weight and food intake were monitored 3 days per week throughout the study. Rats were administered inter-peritoneal injections for 7 days, were allowed to recover for 3 days, and then injected for 3 more days prior to oral glucose tolerance testing with either a prooxidant solution or saline. Six rats received a prooxidant solution of 50 mg/kg doses of both L-buthionine sulfoximine and hydroquinone. Six rats received saline injections. Tail blood was obtained under local anesthesia and tested for glucose using a meter and test strips (One-Touch Ultra). Blood samples were centrifuged and plasma was frozen immediately for later analysis. Oral glucose tolerance testing was conducted after the final day of injections. Rats were fasted 18h then gavaged with a 50% glucose solution at a dose of 6 g/kg. Rats were then injected with either saline or prooxidant solution. Tail blood was obtained under local anesthesia at 0, 30, 60, 120, and 360 minutes after glucose loading. Rat sacrifice with organ harvest occurred within 5 days of oral glucose tolerance testing. Peroxides were measured as a marker of oxidative stress in plasma samples using the ferric orange xylenol (FOX) method (Sigma Chemicals; Peroxi-Detect kit).

## RESULTS

Proxidant injection of L-buthionine sulfoximine and hydroquinone was found to increase blood glucose levels significantly over five days of injections (injected group baseline  $87\pm 5$  to  $234\pm 29$  mg/dL on day 5 versus vehicle group baseline  $96\pm 4$  to  $93\pm 6$  mg/dL on day 5; see Figure 3 on right).



**Figure 3:** Morning blood glucose levels at the beginning of the study were all well below 126 mg/dL in both groups of SHROB (labeled Vehicle Before and O2Stress Before). Glucose levels remained relatively constant and below 126 mg/dL in SHROB receiving vehicle saline injection for 5 days (labeled Vehicle After). In contrast, SHROB subjected to oxidative stress through 5 days of proxidant injections showed marked hyperglycemia, with all but 3 of the 17 rats showing diabetic blood glucose levels above 126 mg/dL.

By the second day of proxidant injection, morning glucose rose significantly above 124 mg/dL, the threshold for diagnosing diabetes. Glucose levels remained elevated for the duration of injection and fell within 3 days upon cessation of injection. However, a re-initiation of proxidant injection in the days leading up to oral glucose tolerance testing resulted in an elevation of blood glucose levels. Control SHROB receiving saline solution remained in the pre-diabetic range (100-125 mg/dL glucose) throughout the course of injections.

Glucose tolerance was found to be impaired in proxidant treated SHROB relative to those receiving saline injection. Following 7 consecutive days of injections, glucose tolerance was measured and found to be impaired in proxidant treated SHROB relative to those receiving saline injection. Oxidative stress significantly increased glucose overall, with blood glucose levels remaining in the diabetic range 360 minutes after glucose challenge. However, upon re-initiation of injections for an additional

3 days following a 3 day recovery period, an oral glucose tolerance test found even greater impairment of glucose tolerance in proxidant treated SHROB relative to those receiving saline injection.

Body weight monitored continuously throughout the study found there to be no change in body weight during proxidant injections as compared to rats receiving control injections. Treatment groups did not differ in body weight by 2-way analysis of variance with repeated measures.

Oxidative stress was measured by assay of peroxides in plasma and the liver fraction of kidney and liver tissue. Proxidant injection increased plasma peroxide levels within 1h with a return to baseline levels within 24h. Baseline plasma peroxide levels were found to be  $20 \pm 2$   $\mu\text{mol/L}$ , with elevation to  $43 \pm 8$   $\mu\text{mol/L}$  at 1h, followed by a subsequent decrease to  $31 \pm 6$   $\mu\text{mol/L}$  at 4h and  $17 \pm 1$   $\mu\text{mol/L}$  at 24h.

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## DISCUSSION

The success of prooxidant treatment in triggering the onset of diabetes in the Spontaneously Hypertensive Obese (SHROB/KoI) rat confirms the association between oxidative stress and the onset of diabetes in a model of metabolic syndrome. Hydroquinone acted to promote oxidative stress through up-regulation of the production of oxidative damage-causing free radicals. L-buthionine sulfoximine, a glutathione synthase inhibitor, served to prevent the break down of harmful free radicals to water and oxygen. The combination of upregulation of free radical production and inhibition of free radical breakdown maximized oxidative stress in the metabolic syndrome-exhibiting SHROB, thus leading to the onset of diabetes. These findings, in conjunction with the fact that saline-injected control SHROB did not develop diabetes, demonstrate that subjection to oxidative stress can cause type 2 diabetes in an at-risk, pre-diabetic model.

The transient elevation of morning blood glucose levels to above 125 mg/dl indicate that the regulation of oxidative damage in SHROB may have a mechanism for short-term recovery from oxidative stress. There are many potential points of regulation of oxidative damage in the SHROB, including the liver, pancreas, and peripheral muscle or fat tissue. Possible mechanisms may include increased glucose production from the liver, impaired insulin secretion from the pancreas, and receptor or post-receptor defects leading to insulin resistance in muscle and fat. Further studies will explore the effect of oxidative stress on each of these possible points of regulation, and seek to determine the possibility of adaptive measures to combat oxidative stress.

The impaired glucose tolerance observed in prooxidant treated SHROB as compared to control SHROB receiving saline injection further demonstrates the effect of oxidative stress in this model. Impaired glucose tolerance is often associated with insulin resistance, a symptom seen in the pre-diabetic SHROB model. However, the further impairment of insulin resistance in the prooxidant treated SHROB indicates the association between oxidative stress and the onset of diabetes.

Obesity is an underlying factor in prediabetes and diabetes. In this study, SHROB body weight remained unchanged despite the increased oxidative stress. This finding rules out the possibility that the onset of diabetes resulted indirectly from increased body weight. The lack of weight loss also suggests that the prooxidant treatment is not simply toxic.

Elevated plasma peroxide levels within 1h of prooxidant injection, with a return to baseline within 24h indicate the activity of internal mechanisms within SHROB for the control of oxidative stress. However, the fact that prooxidant injections administered following a recovery period of three days lead to impaired glucose tolerance, and subsequently higher maximum glucose levels and longer recovery times in an oral glucose tolerance test, may indicate an underlying impairment of SHROB's ability to combat oxidative stress. Subsequent oxidative damage may contribute to insulin resistance in the liver as well as renal and hepatic dysfunction. These are characteristics of chronic type 2 diabetes.

Given the fact that oxidative stress can promote the onset of diabetes in a model of metabolic syndrome, further studies may be performed to explore the mechanism of this association and to investigate preventative treatments for those at risk for developing diabetes. These studies may include the exploration of the effects of diets containing varying levels of anti-oxidants in conjunction with prooxidant treatment, as well as investigation of medications with the potential to inhibit oxidative stress. Anti-oxidant therapies alone or in combination with treatment for characteristics of metabolic syndrome, such as hypertension, may be beneficial in the prevention of the onset of type 2 diabetes in at-risk individuals. This has a widespread potential for benefit due to the increasing frequency of diabetes, obesity, and other characteristics of metabolic syndrome worldwide. Prevention and treatment of these aspects of disease is an important step in the improvement of the overall health of the international population, and future studies of the mechanism of the onset of diabetes through oxidative stress will work towards the achievement of this goal.

## CONCLUSIONS

Induction of oxidative stress in prediabetic SHROB rats causes the onset of diabetes within two days of daily treatment, and further impairs glucose tolerance as measured by oral glucose tolerance testing. Weight gain is not a contributing factor to elevated the blood glucose levels or impaired glucose tolerance seen in prooxidant treated SHROB. Plasma peroxide levels were found to be increased within 1h of prooxidant injection with a return to baseline within 24h. The SHROB rat subjected to oxidative stress is a potential model to test the reduction of oxidative stress and subsequent diabetes prevention by antioxidants in individuals exhibiting metabolic syndrome.

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## FOUR LINES TO IMMORTALITY: DIDO'S RENAISSANCE THROUGH JOSQUIN DES PREZ

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-Dana M. Plank-

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### EDITOR'S NOTE:

To preserve the format of cited passages, this article has been left in a single-column style.

Dulces exuuiae, dum fata deusque sinebat,  
accipite hanc animam meque his exsoluite curis.  
uixi et quem dederat cursum Fortuna peregi,  
et nunc magna mei sub terras ibit imago.

Dear pledges of my love, while Heav'n so pleas'd,  
Receive a soul, of mortal anguish eas'd:

My fatal course is finish'd; and I go,  
A glorious name, among the ghosts below.

(Virgil, *Aeneid*, 4:651-654, trans. John Dryden.)<sup>1</sup>

With these haunting final words, the young queen of Virgil's *Aeneid*, Dido, takes her life on a flaming pyre of her lover's belongings. The death of Dido is one of the most poignant moments in classical literature. Dido begins as an independent queen who rules Carthage without the aid of a male monarch, a model of chastity devoted to her late king. When the hero of *The Aeneid*, Aeneas, arrives in Carthage, Dido is forced by the gods to fall passionately in love with him. In the course of one book, the great queen becomes a suicidal woman broken by love, and a plaything of bickering gods. After proudly resigning herself to this grisly fate, the queen goes on to curse the day Aeneas' ships touched her shores: "From yonder sea may his cold Trojan eyes discern the flames that make me ashes! Be this cruel death his omen as he sails!" (Virgil, *Aeneid*, 4:661-662, trans. Theodore C. Williams). Yet, despite Dido's arresting invective towards Aeneas, the most powerful moment of her speech is not her attack, but her submission to fate.

Many musical works recount Dido's last words; "Dido's Lament" by Purcell comes immediately to mind, as well as motet<sup>2</sup> settings by Alexander Agricola, Jean Mouton, Mabriano de Orto, and Orlando de Lassus, among others.<sup>3</sup> Josquin des Prez's setting of the queen's final speech became the model for a century of imitators. Instead of traversing into the territory of the weeping, livid woman later in the passage, the composer chose to immortalize her quiet acquiescence, setting only the four lines given above. A depiction of the Dido from a few lines later in the text shows her cursing Aeneas' arrival in Carthage and wishing for

her death to become a bad omen for his ships. These cruel words are rife with dramatic tension and musical potential. Josquin's selection of the text, which begins with *Dulces Exuviae* is a powerful indication that he interpreted the maligned queen as a symbol of strength in the face of death. Josquin's interpretation of the text became a powerful model for later composers, and his choice of text allowed Dido to go "to the ghosts below" not as a madwoman queen, but as a glorious name amidst the timeless characters of classical literature. (Dryden)

Despite the fact that Dido was best known in the Renaissance as a character, she was not an entirely fictional creation. There are many sources elaborating on the true queen. The real Dido was a Tyrian princess at the end of the 8<sup>th</sup> century B.C., known for her chastity and unwavering devotion to her slain king. When her husband was killed, Dido threw herself into service of her people, vowing never to remarry. However, she was soon pressured into a second marriage, and committed suicide so as not to betray the memory of her first husband (Schmalfeldt, 584).

Virgil altered the story for the sake of art. The poetic Dido is placed more than three hundred years before the real queen lived. Virgil changed the dates to make Dido's rule coincide with the end of the Trojan War in the 12<sup>th</sup> century B.C. (Ibid, 585). In Virgil, Aeneas is driven to the shores of Carthage by the Fates' meddling. Aeneas is the son of the goddess Venus, who is intent on destroying all obstacles to her son's realization of his destiny. As Venus is obviously well-acquainted with the consequences of unrequited love, she orders Amor (Cupid) to "enflame [Dido's] heart with a passion for Aeneas that is uncontrollable and ruinous." (Ibid, 585). This divine intervention turns the chaste queen into a lustful temptress who offers Aeneas rule of her kingdom in exchange for his love.

The apex of the fourth book is an episode in which Dido's misunderstanding begins the downward spiral that will eventually result in her death. The gods cause a horrible storm during a hunting party, forcing Aeneas and Dido to seek shelter in a cave for the night. That night, Dido breaks her vow of chastity. The love-struck queen believes that this union is a consummation of marriage, but Aeneas is bound by Fate to reject this contract and departs with his men. The queen is inconsolable at the realization that she has ruined herself, broken her vows to her dead husband, and abandoned her city for a shameful tryst. Her only solution is to cover her indignity with a swift and dramatic suicide atop a fire of Aeneas' belongings. She throws herself on the sword of her former lover, surrounded by flames.

The real Dido did kill herself, but the addition of a fire was a new one in literary tradition. Women in mythology often took their own lives by hanging, poison, and the sword (Edgeworth, 129). The fire was a dramatic and unusual addition to the story. Ancient historians Polybius and Appian both wrote accounts of the fall of Carthage, and it is in their writings that one finds the basis for Virgil's Dido (Edgeworth, 131). Virgil did his research on Carthage; Polybius tells of the death of the anonymous final queen of Carthage in 146 B.C. The unnamed woman was married to the Carthaginian commander Hasdrubel, and when the city was seized, he willingly surrendered to Africanus. Reportedly, Hasdrubel's wife walked out of the temple of Eshmoun with her young children in tow. She set fire to the building and reproached her husband for his shameful capitulation (Edgeworth, 131). Appian attributes a rather dramatic final speech to the historical queen:

'Wretch,' she exclaimed, 'traitor, most effeminate of men, this fire will entomb me and my children. But as for you, what Roman triumph will you, the leader of great Carthage, decorate? Ah, what punishment will you not receive from him at whose feet you are now sitting?'<sup>4</sup>

After scolding her own husband for his submission to the enemy, the anonymous queen submits herself and her children to the flaming temple.

The death of Dido is heavily symbolic in the scope of Virgil's epic; Dido's love has caused not only her undoing but the fall of her beloved city as well. The flames of Dido's funeral pyre represent her inner turmoil caused by her doomed relationship with Aeneas. The queen had a powerful will that first attracted Aeneas and a sense of duty to her people that is meant to mirror Aeneas' end goal of founding the great Roman race. Dido is a puppet of the gods, enflamed with love in order to derail the young hero from his destiny. From there Dido descends into rage, reproach for Aeneas, and her eventual death aboard the funeral pyre.<sup>5</sup>

The dichotomy between the historical and fictional Dido is the difference between a martyr for chastity and a timeless romantic tragedy. While the unsullied queen may have been a powerful moral example, the impure Dido becomes

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a timeless inspiration. Her side of the story inspired immediate sympathy; Ovid wrote a collection of fictional responses from the scorned women in mythology called the *Heroides*, each written as a letter in the lyrical elegiac couplet. The seventh book contains a large letter from Dido to Aeneas.<sup>6</sup> Ovid's Dido is increasingly sympathetic; her letter moves from disparaging to reproachful to pathetic at times. She begins by stating that the letter is not meant to win Aeneas back:

Not because I hope that you can be moved by my prayers  
Do I speak--I speak them with the gods against me;  
But having lost, miserably, my merit and reputation, my virtue  
Of body and spirit, to lose words is a small thing (trans. Hunter, lines 3-6).

Yet Dido's letter is full of intent. She wants to curse Aeneas; she wants to reflect on her mistakes and her impending suicide. She is sarcastic: "Are you determined, Aeneas, to release your promises with your ships," (Ibid, line 9), She wishes a sweeter revenge than her false lover's swift death:

Live, I pray you. Thus I shall destroy you more fully than by death.  
Rather, you shall be said to be the cause of my death.  
Consider that you are seized--may there be no weight to the omen--  
By a fierce storm; what will be in your mind?  
Immediately will come the perjury of your false tongue,  
And Dido driven to die by Phrygian<sup>7</sup> deception (Ibid, 63-78).

The queen speaks extensively of what she has given up for her foolish love. She tells of the suspicious death of her husband, and her journey to distant soil to found Carthage. She speaks of the wars and ardent suitors that assailed her because she was a foreign woman leading a prosperous city: "I purchase this shore, traitor, which I gave to you. I founded a city, and set down extensive walls." (Ibid, 118-119).

Ovid's Dido even speaks of scandal-- the potential that more than one life could be taken up by the flames:

Perhaps also it is a pregnant Dido, evil one, whom you abandon,  
And a part of me lies hidden in my body.  
The wretched infant will join the fate of the mother,  
And you will be the author of the death of your unborn child (Ibid, 138-143).

Her closing words after this invective diatribe are powerful: "If you are ashamed of me as a wife, then let me be called not bride but hostess; So long as she is yours, Dido will be what you wish." (Ibid, 172-173).

Ovid's Dido is more emotionally unstable than Virgil's. Her letter changes moods frequently and describes an incredibly bitter fallen queen. The example, however, serves to illuminate Virgil's importance. His episode with Dido has changed from a side quest in the greater scheme of Aeneas' epic to a work of art in itself. Many works since have recreated the monarch's internal monologue, fraught with strife and reproach for the supposed hero of the epic. This tradition of sympathy extended well into the late 16<sup>th</sup> century in musical settings as well as literary interpretation.

The practice of setting Latin classical texts as motets is not uncommon in musical history. The form of the motet was originally derived from liturgical chant accented with secular poetry. The secular text was considered an allegorical elaboration of the themes in the sacred text (Randel, 529). While the motet is generally thought of as a genre sacred to Christianity, Tinctoris' 1495 dictionary of musical terminology states that a motet could be on any subject, though sacred texts were the most common (Randel, 530). Thus, one could make exceptions for secular texts from classical Latin poetry.

Fifteenth century education relied on the ancient Roman epics as teaching tools. Albert Seay, a musicology professor at Colorado College, wrote on medieval exposure to classical texts and the use of classical metrics in music. He states:

The study of the three great Golden Age poets (and others as well) remained as one of the strongest parts of the school curriculum, with every student eventually taken through the classics. With the renewed interest in the classics during the fifteenth century and with the beginnings of printing as a way of diffusion of texts, the audience for the Latin classics (and the Greek ones as well) became a wide one indeed...(Seay, 64).

Furthermore, historical sources in Seay show that the memorization of classical poetry was aided by the use of song. Seay documents the symbiosis of music and Latin education:

In teaching the classics, music was called into service as a valuable instructional aid. Instead of merely reciting those sections of *the Aeneid* that one had learned by straight memorization, some devised simple ways of singing them, so that they would be easier to recall (IBID, 64).

In the late 15<sup>th</sup> century composers would have been indoctrinated into the association of music with classical Latin. Tinctoris makes mention of Virgil, Ovid, or Horace in all twenty chapters of his treatise *Complex of the Effects of Music* (IBID, 64). Perhaps it was not such a stretch of the imagination for a composer to utilize these highly elevated texts in pursuit of artistic expression.

Dido became a popular subject in Renaissance music. The text that gained the greatest prominence was derived from the queen's final speech in book four of the *Aeneid*. While the speech is rife with inflammatory passages and stately language, the four lines that were singled out for musical setting by Josquin des Prez became emblematic of Dido's entire saga. The original Latin is given above.

It is useful to compare several translations in order to understand these lines fully. John Dryden's translation, given above, attempts to make English poetry of the Latin text. Dryden utilizes rhyme and iambic pentameter in his translation, which indicates that he was not simply aiming to translate the Latin text for his readers. He attempts to create English poetry as rich and meaningful as the original Latin. His ability to recast the original text demonstrates a sophisticated understanding of the poetry, and his version is to be applauded for its individual artistic endeavor.

A second translation by Theodore Williams is a more literal treatment, though the language is still lofty.

Sweet relics! Ever dear when Fate and Heaven  
upon me smiled, receive my parting breath,  
and from my woe set free! My life is done.  
I have accomplished what my lot allowed;  
and now my spirit to the world of death  
in royal honor goes (Trans. Williams, lines 651-654).

This translation follows the word order as it appears in the Latin. Even though the English seems to be slightly out of place, this translation demonstrates that Virgil writes carefully to the meter, arranging the words so that they fit the dactylic hexameter<sup>8</sup> scheme used in all Roman epic poetry. A more literal translation might have read: "and now in royal honor, my spirit goes to the world of death."

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Nigel Coulton's translation is particularly helpful in analyzing musical examples of the *Dulces Exuviae*. His translation is given as part of in the introduction to an edition of five Virgil motet settings from the fifteenth and sixteenth centuries. This translation utilizes plain language and follows the Latin progression directly:

What he has left behind, sweet to me while fate and god allowed,  
take this my life and release me from these my woes.

I have lived a life and completed the journey that fate gave me,  
and now my proud spirit will go beneath the earth (Trans. Coulton, lines 651-654).

Some of the most important features of these four lines are the in nature of Dido's words. Her tone is calm for a woman about to impale herself on her former lover's sword. Regardless of how she fell in love with Aeneas, Dido still dies a queen. She realizes that her destiny is not her own, but dominated by the whims of Fate as stated in the line that reads "uixi et quem dederat cursum Fortuna peregi."

Oliver Strunk, a founding member of the American Musicological Society who is a prominent editor and writer for the society's journal, suggests that this section lends itself to a motet setting because the four lines are complete in themselves. Longer passages would have been far more cumbersome to set to music (Strunk, 488). The composer's poetic association of a tragic queen going solemnly to her death is a poignant text that is easy to manipulate with musical devices for heightened emotional impact. This sentiment is echoed by Leofranc Holford-Strevens, a prominent Renaissance scholar, as well: "Dido's lament seems to draw the composers in because her final words are not simply broken and love-struck, but full of pride and a resignation to fate." (Holford-Strevens, 371).

The University of Florida's motet database lists 26 motets from the late 14<sup>th</sup> through the 15<sup>th</sup> and 16<sup>th</sup> centuries (Thomas).<sup>9</sup> Composers from Josquin des Prez to Orlando di Lasso are represented. In nearly all of the examples, the motets consist of the four lines beginning with "dulces exuviae." Only Orlando di Lasso in his 1570 setting extends the text to include more of the speech (Guentner, 66). The Josquin setting uses lines 651-654; Lasso continues on to line 660. The resulting composition is far longer than any of the other examples, but it is also one of the last incarnations of the *Dulces exuviae* motet.

Josquin's setting of the *Dulces exuviae* is attributed to the composer in a manuscript from Brussels/Mechelen dated between 1516-1522 (Thomas). This makes Josquin's one of the earliest extant settings of the text. Josquin's work is highly attentive to the meaning of the Latin, and his overwhelming emphasis seems to be the repetition of important words and phrases and imitative gestures between parts. The motet does not give rhythmic emphasis to support the dactylic hexameter of the original poetry (Guentner, 64). However, the piece's structure is carefully organized by the Latin text; each major cadence<sup>10</sup> occurs at the end of a poetic line, and each new point of imitation occurs at the beginning of a poetic line. Josquin's compositional style in this motet is centered around the clarity and meaning of the text rather than complicated melodic innovations.

As the text is so short, Josquin divides many of the poetic lines into two units. The effect of this division is maintained clarity of text declamation in spite of the imitative horizontal motion. However, Josquin reserves prominent cadences (with authentic, or 5-1 bass motion) for the true ends of poetic lines. The strongest cadences occur at m.14 ("sinebat," the end of the first poetic line), m.22 ("curis," the end of line 2), m.33 (on "peregi," the end of line 3), and the final, drawn-out cadence in the last measure. The Dorian cadence at m.14 comes to a complete stop before moving to "accipite hanc" in m.15. The large cadence in m.22 (on "curis," also in Dorian) has a similar sensation of coming to a complete rest before continuing. While there are passing notes in the alto part, they come to rest in m.23 before the half note motion on "vixi et quem."

One of the most striking cadences occurs at the end of the third line, in measure thirty three, on the words "magna mei." The soprano line has an ascending motion to the cadence which seems to highlight Dido's pride. The words "magna mei," which refer to her great name, and the ascending music seems to give the word a majestic emphasis. The bass also moves in a 2-1 motion instead of the stronger 5-1. However, this cadence occurs in the middle of a poetic line, and so the 2-1 motion is likely intentional. The beginning of the piece has a few smaller cadential gestures at the midpoints of lines,

such as m. 10 (in the middle of “dum fata”) and m.12 (in the middle of “deusque”), but these gestures are not as strong as the other cadences because one of the voices immediately moves to the next note or word of text.

The final cadence is particularly striking. The piece ends on a Phrygian cadence while the other cadences of the piece are predominantly in Dorian. The 2-1 motion of the bass lacks the strength of a 5-1, and the soprano does not rest on the final note of G, but ascends in the second half of the bar to a B. This rise gives the final measure an open-ended sound, and the ascending note contrasts with the numerous descending figures that appear before it. The last note gives a sense of ambiguity to what should be the final resolution. Because it follows a piece characterized by calm pride, this last measure's uncertainty lends a subtle drama to Dido's plight. The open-ended nature implies questions without answers, life without understanding, death without resolution.

Despite Josquin's frequent use of imitation and repetition (particularly in the first two lines of the poem), the four voices are rarely more than a measure apart in their declamation of the text. Often, the ends of poetic lines are clearly marked with long note values, and other voices begin the imitation anew over the previously held note. A good example is in the middle of the first line of text. In m.8 of Bernard Thomas's edition of the piece<sup>11</sup>, the soprano line holds a G on the “ae” of “exuviae” for the length of a double whole note while the other three voices move on to “dum fata” in half and quarter notes.

A particularly striking entrance occurs in the second line of poetry, on “accipite hanc,” where Dido implores death to receive her life. The rhythm seems to fit the word particularly well; “accipite” is declaimed on a half note, two quarter notes, and another half note in m. 15, and carried through all four voices. It is an interesting contrast from the long values of the first entrance on “dulces,” which is written mostly in whole notes and double whole notes. The alto and bass lines declaim the text together on this striking rhythm. This effect is imitated in m. 30, where the soprano and tenor lines have the same eighth-note rhythm on “peregi.” This echoes the previous settings of “accipite” in the other two voices, and shows Josquin's attention to vocal groupings. His setting of *Dulces exuviae* may not be chordal, but he is by no means ignorant of the vertical interaction of his vocal lines. His attention to rhythmic affect is a source of beauty in the music.

No voice sings the exact poem in its unadulterated form. Each voice has repetitions of words or entire phrases. Other settings of the text assign the entire poem in at least one voice.<sup>12</sup> De Orto chooses to place the pure line in the soprano, and the anonymous setting from the Bernard Thomas edition places the unadorned version in the tenor. This lack of an untouched line in Josquin highlights certain words of the text.

One such word is “cursum” (mm. 25-28). All four voices have overlapping melismas<sup>13</sup> on this word, which means “course” or “journey.” This is a vivid example of text painting, as the winding lines imply the winding course of the life Dido has lived. A melisma is a musical journey, and a fitting device for demonstrating the difficult path Dido has had to travel. Josquin's voices each have unique patterns of repetition. Whether these repetitions were simply for vertical alignment or for word emphasis, they give weight to certain words of the short text. The soprano, for example, repeats the words “dulces exuviae” and “sinebat” twice each, emphasizing the belongings that represent the last vestige of her lover and an active memory of the past. “Sinebat” is in the imperfect tense, a tense for habitual past action. She is reflecting on the items Aeneas left behind, and the brevity of her affair “sweet to me while fate and god allowed” with the future founder of Rome (trans. Coulton).

The alto also focuses on the introductory material of the poem. The line repeats “dulces exuviae” (sweet relics) twice, “dum fata” (while Fate) three times, “deusque” (and God) twice, and “curis” (woes) twice. The “dulces exuviae,” “dum fata,” and “deusque” are all extracted from the first line of the poem. The second voice emphasizes the two forces that caused Dido's love of Aeneas: “fata” (Fate) and “deusque” (and God). The alto becomes slightly mournful in its repetition of “curis” (woes). The alto emphasizes the queen's wish for release from earthly anguish. The bass line repeats “exuviae” twice and “dum fata” twice. This pattern, coupled with the voices above it, shows a stronger sense of repetition in the beginning of the poem more traditional declamation at the end. The second, third, and first half of the fourth poetic lines rely more on melismatic material than on simple repetition.

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One of the most arresting aspects of the piece is Josquin's treatment of the final words "sub terras ibit imago" (I will go beneath the Earth, line 654). The soprano repeats "sub terras ibit imago" three times, followed by two repetitions of the "ibit imago," which gives the sensation of fading into her accepted fate (mm. 34-43). The alto repeats "sub terras ibit imago" twice, followed by four repetitions of "ibit imago," which reinforces the feeling of the soprano (mm. 33-43). The tenor line is an even weaker echo; it does not repeat the whole phrase but begins with three repetitions of the "ibit imago" and fades to four repetitions of the "imago" (mm. 34-43).

The bass makes its most adamant word repetitions at the end of the motet. Copied exactly from the Bernard Thomas edition (mm. 32-43), it reads: "sub terras, sub terras, sub terras ibit, sub terras ibit imago, sub terras ibit imago, ibit imago." The bass has the most repetitions of the phrase, begins the line the earliest, and sings it in the lowest range. The effect of the bass' repetition pattern is to cause suspense. The full line is not revealed until its fourth iteration in mm. 37-39. There is a sense of reluctance in its unwillingness to provide the entire text at first. It undermines Dido's resolve. Perhaps there is a twinge of doubt. Perhaps she is slightly afraid of her death and is stuttering to get the words out. This phrase echoes again and again in the voices, the way it might sound in a cave if spoken aloud. The repetition and descending note patterns enforce the finality of Dido's words as she announces her death. Whether or not the queen is afraid to die, she has decided to do so, and this fitting close to the motet reflects her persistence. Perhaps the repetitions are a source of strength; a method of steeling herself for her encounter with Aeneas' sword.

Josquin's *Dulces Exuviae* does not clutter the text with over-wrought counterpoint<sup>14</sup>. The spare quality to the echoed interactions between lines is quite arresting. The voices move with such calculated intervals through the text that one hears Josquin's emotional interpretation of the classical text. Dido's words gain a quietly haunting quality through this motet that can only be fully realized through performance. There is a slow, stately feeling to the piece, even when note values diminish from double-whole notes to eighth notes. There is a sense of broadening calm and contemplation. Dido's words are expressed with the grace of one of the finest composers of the Renaissance.

Josquin may never have chosen to set Dido's speech had Virgil's poetry not been such a ubiquitous teaching tool for the Latin language. Classical poetry was the gateway to the Latin of the Church. The use of the great Roman epics in the classroom provided a venerable canon of works, along with the liturgy, that could serve as an acceptable source of artistic inspiration for Renaissance composers. The study of these epics alongside Biblical texts legitimized their allegorical connection to the liturgical works. However, Virgil's rebirth in the classroom was fueled not only by the strength of his poetry, but also by others viewing him as a prophet of Christ.

One reason for the Church's adoption of Virgil appears in the Fourth Eclogue<sup>15</sup>, written in 40 B.C.<sup>16</sup> The beginnings of this allegorical intervention seem to stem from Emperor Constantine in 324 A.D. His quotation of the Eclogue gave the work prominence in the newly Christian empire. The language of the Fourth Eclogue<sup>17</sup> is lofty and somewhat vague, yet its message is well suited for Christian interpretation. Virgil states early in the work that the birth of one child will change the course of humanity, that a great race shall arise from the arrival of this sacred child. A translation of the Latin states:

Justice returns, returns old Saturn's reign,  
With a new breed of men sent down from heaven.  
Only do thou, at the boy's birth in whom  
The iron shall cease, the golden race arise (Trans. MacKail, lines 8-11).

Men of the Catholic Church saw themselves as this new race whose golden age was ushered in by the birth of Jesus.

The Eclogue elaborates on the blessings brought forth by child's birth. The poem speaks of gods and men living harmoniously, a fertile and abundant earth, and an age of great joy and wisdom under the rule of the child and his father. It even suggests Christ's ability to forgive sin.

Under thy guidance, whatso tracks remain  
Of our old wickedness, once done away,  
Shall free the earth from never-ceasing fear.

The holy nature of the child and his relationship with divinity, particularly his father, is also clearly stated.

He shall receive the life of gods, and see  
Heroes with gods commingling, and himself  
Be seen of them, and with his father's worth  
Reign o'er a world at peace (trans. MacKail, lines 19-22).

Parallels to Biblical imagery can be easily drawn from the text; even the serpent from the Garden of Eden seems to make an appearance: "The serpent too shall die." (Ibid, line 31). Because the serpent's death is mentioned among the many results of the boy's birth, it also indicates a significant parallel to the triumph of Christ over evil. Because the poem is written in flowery prose, pastoral references and suggestions of divinity cause the Church to see Virgil's Eclogue as a reverent prophecy of Christ.

Modern Classical scholars have attributed the Eclogue to more plausible subjects than the future Messiah. An heir of Octavian, Marc Anthony, or Virgil's patron, Pollio, could have been the original subject of the poem (Townend, 70). Yet the similarity of Virgil's predictive language to Biblical accounts was particularly striking to medieval readers, and thus the Church elevated him from poet to prophet (Thompson, 648).

Beyond the poetry itself, association with some of the most highly revered figures in the Church gave Virgil a place of prominence. St. Augustine recounts in his *Confessions* his experience reading the *Aeneid* and how he was moved by the fourth book (Schmalfeldt, 615)<sup>18</sup>. St. Augustine speaks extensively of his education in Carthage, and the impact of his encounters with the great Greek and Roman poets. He chastises himself for his secular pity for Dido for several paragraphs.

Who is more pitiful than a pitiable man without pity for himself—one who weeps for Dido, dead because she loved Aeneas, but not for himself, dead because he failed to love you, God, my heart's enlightener, the feeder of my soul's inner hunger, the vital principle breeding depth of thought out of my intelligence? I was the abandoner, the faithless lover, and my faithlessness earned the world's Bravo! Bravo!—since love of the world is abandonment of you, and the world cries Bravo! Bravo! to keep its own in line. For all this I had no tears, only tears for Dido, exploring with the sword her utmost doom (Augustine, *Confessions*, trans. Wills, 16).

Virgil also appears extensively in the *Divine Comedy*, Dante Alighieri's fourteenth century epic poem about a Christian soul's journey towards salvation. Virgil appears as a spiritual guide through hell and purgatory. Dante so respected Virgil that he is presented as a guiding force in a work centered on Christian themes. Despite the writer's obvious admiration for his pagan predecessor, Dante was well aware of the boundaries; the polytheistic poet cannot pass into Paradise. Even viewed as a prophet of Christ, Virgil still had the impurities of his former religion to contend with (Hollander, 2)

So why is Dido such a lasting figure? After all, as Schmalfeldt writes, "...it was for Dido, not Aeneas, that Augustine wept." (Schmalfeldt, 593). If *The Aeneid* were reinterpreted to allegorically represent the journey of the soul towards Christian salvation, then what part does Dido play? Much of book four undermines a Christian interpretation of *The Aeneid*, because she breaks a long standing vow of chastity. Should Dido not be resigned to the role of Aeneas' temptress, loathed for her manipulation of the pure hero? Christian teachings forbid illicit sex out of wedlock, and both of Virgil's characters are guilty of lust.

Virgil's Dido is not a reformed temptress. She does not succeed in seducing Aeneas into staying in Carthage and becoming her king, so, she takes her own life so as to escape her broken vow and her shame. Dido chooses not to atone for her sins in a Christian manner; she commits suicide, one of the most abhorrent sins in Christianity. If anything, Dido's role is to undermine Aeneas' purity; he sleeps with the formerly chaste queen, yet has no intention of remaining in Carthage to care for her as a second husband.

It appears that the continued fascination with the Carthaginian queen was due to the unresolved tensions in Virgil's treatment of Dido with respect to Aeneas' quest. There is an aspect of cruelty in Aeneas' treatment of Dido; yet all is forgiven in the name of destiny. Aeneas' shortcomings are accepted as divine intervention and gods' attempts to



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interfere with destiny. The queen is simply a pawn of meddling deities; however, the supposedly pious Aeneas could have refused her love the night of the storm. While Aeneas continues his quest with no consequences for his actions, Dido is left dead at the end of the fourth book. Dido is a victim of Aeneas and gods, and yet she is the one who loses her life. Her love was not true; it was induced. Perhaps she would never have fallen for Aeneas if Amor had not intervened.

Dido's apparent lack of free will suggests an interesting interpretation of her character. If one looks upon the queen as a miserable pawn in Aeneas' destiny, meant to derail his search for Rome by vindictive deities, then her story becomes sympathetic. Dido becomes a victim of the gods in a game gone horribly awry. Thus, the true Dido is a strong, chaste woman ruling her city without a husband, before she is led astray by lust. It is this victimization that seems to have inspired Renaissance characterizations of Dido as the pure queen brought to ruin by Fate.

Dido is not only a pawn of the gods; she is also used as a poetic tool, meant only to serve as Aeneas' foil and display how his destiny could fall apart through love. She is the mirror of Aeneas; after all, "Both are widowed and in exile, both are obeying commands to found a new city and serve as its magistrate..." (Schmalfeldt, 588). Dido represents everything he is meant to achieve as the fated ruler of a thriving city.<sup>19</sup> This aspect shows her strength and the source of her pride.

Virgil, for all of his poetic genius and classical hexameters, might never have imagined a future in which he would be hailed as a prophet of a Messiah or an inspiration for dozens of musical compositions. While Virgil's poetry glorifies the ideals and virtues of his native Roman Empire, the artistic resonance of Dido has been felt across disciplines for centuries. Yet without the reception of his texts as Christian allegory, the fascination with Virgil might never have occurred in the middle ages. Sacred interpretation is what caused the Aeneid to be canonized in the fifteenth century, and this reevaluation of the epic allowed Dido to be reborn as an unwitting victim and not relegated to the role of goddess temptress.

Virgil became a symbol of Christian prophecy. Likewise, his works gained new life centuries later in motet settings of his poetry. This shift is where the true value of the epic becomes apparent. Through fictionalization, Dido evolves into a contemporary of Aeneas, temporally displaced three hundred years to the aftermath of the Trojan War. Her kingdom symbolizes the destruction of love at the hands of the gods, the incredible sacrifice that the deities are willing to make for the life of one man. The sacrifice of the gods is not simply one human life, but the death of an entire civilization for the sake of the Roman race. Dido has become a tragic heroine, monolithic symbol far beyond the flesh.

Ironically, Virgil gained immortality in the Renaissance not through his hero Aeneas, but through a broken queen, a victim of fate and spurned love. Dido's farewell imparts a melancholy journey to the realm of the dead which inspired Josquin and subsequent composers to set her final speech to music. Josquin's motet interpreted Dido not as one of the many spurned women of antiquity, but as a proud symbol of acceptance. The queen's final words become evocative subjects for musical invention, and Josquin's emphasis on repetition and clarity of text declamation demonstrate a reverence for the meaning of the words. For all of the viable poetry in book four of the Aeneid, Josquin's chose to single out four lines that both immortalized Dido in music and portrayed her as a wronged heroine. *The Aeneid* thus became a permanent source for musical allusion that ensures that Dido will eternally go to her grave with her proud name intact.

### Appendix One

#### Passages Surrounding the Dulces Exuviae with Dryden and Williams Translations

Dardanium, non hos quaesitum munus in usus.  
hic, postquam Iliacas uestis notumque cubile  
conspexit, paulum lacrimis et mente morata  
incubuitque toro dixitque nouissima uerba: 650  
‘dulces exuuias, dum fata deusque sinebat,  
accipite hanc animam meque his exsoluite curis.  
uixi et quem dederat cursum Fortuna peregi,  
et nunc magna mei sub terras ibit imago.  
urbem praeclaram statui, mea moenia uidi, 655  
ulta uirum poenas inimico a fratre recepi,  
felix, heu nimium felix, si litora tantum  
numquam Dardaniae tetigissent nostra carinae.’  
dixit, et os impressa toro ‘moriemur inultae,  
sed moriamur’ ait. ‘sic, sic iuuat ire sub umbras. 660  
hauriat hunc oculis ignem crudelis ab alto  
Dardanus, et nostrae secum ferat omina mortis.’

Then swiftly to the fatal place she pass'd,  
And mounts the fun'ral pile with furious haste;  
Unsheathes the sword the Trojan left behind  
(Not for so dire an enterprise design'd).  
But when she view'd the garments loosely spread,  
Which once he wore, and saw the conscious bed,  
She paus'd, and with a sigh the robes embrac'd;  
Then on the couch her trembling body cast,  
Repress'd the ready tears, and spoke her last:  
“Dear pledges of my love, while Heav'n so pleas'd,  
Receive a soul, of mortal anguish eas'd:  
My fatal course is finish'd; and I go,  
A glorious name, among the ghosts below.  
A lofty city by my hands is rais'd,  
Pygmalion punish'd, and my lord appeas'd.  
What could my fortune have afforded more,  
Had the false Trojan never touch'd my shore!”  
Then kiss'd the couch; and, “Must I die,” she said,  
“And unreveng'd? 'T is doubly to be dead!  
Yet ev'n this death with pleasure I receive:  
On any terms, 't is better than to live.  
These flames, from far, may the false Trojan view;  
These boding omens his base flight pursue!” (Dryden Translation)

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There she leaped,  
a frenzied creature, on the lofty pyre  
and drew the Trojan's sword; a gift not asked  
for use like this! When now she saw the garb  
of Ilian fashion, and the nuptial couch  
she knew too well, she lingered yet awhile  
for memory and tears, and, falling prone  
on that cold bed, outpoured a last farewell:

“Sweet relics! Ever dear when Fate and Heaven  
upon me smiled, receive my parting breath,  
and from my woe set free! My life is done.  
I have accomplished what my lot allowed;  
and now my spirit to the world of death  
in royal honor goes. The founder I  
of yonder noble city, I have seen  
walls at my bidding rise. I was avenged  
for my slain husband: I chastised the crimes  
of our injurious brother. Woe is me!  
Blest had I been, beyond deserving blest,  
if but the Trojan galleys ne'er had moored  
upon my kingdom's bound!”

So saying, she pressed  
one last kiss on the couch. “Though for my death  
no vengeance fall, O, give me death!” she cried.  
“O thus! O thus! it is my will to take  
the journey to the dark. From yonder sea  
may his cold Trojan eyes discern the flames  
that make me ashes! Be this cruel death  
his omen as he sails!” (Theodore C. Williams translation)

## Appendix Two

## MOTET Online Database listings for Dulces Exuviae

#	Composer	Source	Ed. date	Publisher or Scribe	City	Country
1	Agricola, Alexander	LonBLR 8 G. vii	1516-22	Alamire, Petrus	Brussels/Mechelen	Belgium
2	Desprez, Josquin	LonBLR 8 G. vii	1516-22	Alamire, Petrus	Brussels/Mechelen	Belgium
3	Mouton, Jean	LonBLR 8 G. vii	1516-22	Alamire, Petrus	Brussels/Mechelen	Belgium
4	Johannes Ghiselin	LonBLR 8 G. vii	1516-22	Alamire, Petrus	Brussels/Mechelen	Belgium
5		LonBLR 8 G. vii	1516-22	Alamire, Petrus	Brussels/Mechelen	Belgium
6	Willaert, Adrian	MunBS 274a	1540-60, c.		Augsburg	Germany
7	Peschin, Gregor	RegB B220-2	1540-60, c.		Salzburg?	Austria
8		BrusBR 228	1519, c.	Neth Ct. "C" or "X"	Brussels/Mechelen	Belgium
9	Orto, Mabrianus de	BrusBR 228	1519, c.	Neth Ct. "C" or "X"	Brussels/Mechelen	Belgium
10	Ghiselin, Johannes	Selectissimae necnon fam. cant.	1540/7	Kriesstein	Augsburg	Germany
11	Willaert/Mouton	Tricinia. Tum veterum tum recentiorum in arte musica symphonistarum	1542/8	Rhau	Wittenberg	Germany
12	Ghiselin, Johannes	WittenL 1048	1524-38			Germany
13	Freminot	BergBC 1209	1545	Albertis, Gaspar Ulhard		Germany (Bavaria)
14	Willaert, Adrian	Concentus 8, 6, 5, 4 vc, omnium iucundissimi	1545/2	Ulhard	Augsburg	Germany
15	Mudarra, Alonso	Alonso Mudarra: Tres libros de musica en cifras para vihuela		Juan de Leon	Seville	Spain
16	Willaert, Adrian	Sacrarum cantionum 4 vc Bk 3	1547/5	Susato	Antwerp	Belgium
17	Willaert/Mouton	RegB 940-1	1557-59	Wolfgang Küffer + Heugel, Johann Berg & Neuber	Regensburg/Wittenburg	Germany (Bavaria)
18	Brätel, Ulrich	KasL 38	1535-66 in MS	Heugel, Johann Berg & Neuber	Kassel	Germany
19	Desprez, Josquin	Tertia pars magni operis musici	1559/2	Berg & Neuber	Nürnberg	Germany
20	Mouton, Jean	Tertia pars magni operis musici	1559/2	Berg & Neuber	Nürnberg	Germany
21	Lassus, Orlande de	MunBS 20	1550, post			Germany
22	Willaert/Mouton	Symphoniae iucundae atque adeo breves quatuor vocum	1538/8	Rhau	Wittenberg	Germany
23	Agricola, Alexander	AugsS 142a	1505-14		Augsburg	Germany
24	Orto, Mabrianus de	FlorC 2439	1506-14	Bourgeois, Martin?	Brussels/Mechelen	Belgium
25	Willaert/Mouton	SGallS 463 (Tschudi Liederbuch)	1540 ff	Tschudi, Aegidius	Glarus	Switzerland
26	Ghiselin, Johannes	SGallS 463 (Tschudi Liederbuch)	1540 ff	Tschudi, Aegidius	Glarus	Switzerland

Appendix Three

Bernard Thomas' Edition of the *Dulces exuviae* by Josquin des Prez

5. DULCES EXUVIAE III

Josquin des Prez

Dul - ces ex - u - vi - ae, dul -  
Dul - ces.  
Dul - ces ex - u - vi - ae,  
ces ex - u - vi - ae,  
ces ex - u - vi - ae, dum fa - ta dum fa -  
ex - u - vi - ae, dum fa -  
ex - u - vi - ae, dum fa - ta, dum fa -  
dum fa - ta de - us - que si - ne -  
- ta, dum fa - ta de - us - que, de -  
- ta, de - us - que, de - us -  
- ta, de - us -  
bat, si - ne - bat, ac - ci - pi - te hanc  
- us - que si - ne - bat, ac - ci - pi - te hanc a - ni -  
que si - ne - bat, ac - ci - pi - te hanc a - ni -  
que si - ne - bat, ac - ac - pi - te hanc

LPM 551

11  
a - ni - mam me - que his ex - sol vi -  
mam me - que his ex - sol - vi -  
mam me - que his ex - sol - vi - te  
mam ex sol - vi - te cu -

21  
te cu - ris, Vi - xi  
te - cu - ris, cu - ris, Vi - xi quem de - de -  
cu - ris, Vi - xi et  
ris, Vi - xi et quem

25  
et quem de - de - rat cur -  
-rat cur - sum  
quem de - de - rat cur -  
de - de - rat cur -

28  
sum for - tu - na per - e -  
for - tu - na, pe - re -  
sum for - tu - na, per - e -  
sum for - tu - na, per - e - gi

LPM 551

-gi, et nunc mag - na me - i  
 -gi, et nunc mag - na me - i sub ter -  
 gi, et nunc mag - na - me - i  
 et nunc mag - na me - i sub ter - ras, sub ter

34  
 sub ter - ras i - bit i - ma -  
 ras i - bit i - ma - go, sub ter -  
 sub ter - ras i - bit i - ma -  
 ras, sub ter - ras i - bit i - ma -

37  
 go, sub ter - ras i - bit i - ma -  
 ras i - bit, i - ma - go, i -  
 go, i - ma - go, i - bit i - ma -  
 bit, sub ter - ras i - bit i - ma - go, sub ter - ras

40  
 go, i - bit i - ma - go.  
 bit i - ma - go, i - bit i - ma - go.  
 go, i - bit i - ma - go.  
 i - bit i - ma - go, i - bit i - ma - go.

LPM 551

## Appendix Four

Virgil's Fourth Eclogue

Translated J.W. MacKail

### POLLIO

Muses of Sicily, essay we now  
A somewhat loftier task! Not all men love  
Coppice or lowly tamarisk: sing we woods,  
Woods worthy of a Consul let them be.  
Now the last age by Cumae's Sibyl sung  
Has come and gone, and the majestic roll  
Of circling centuries begins anew:  
Justice returns, returns old Saturn's reign,  
With a new breed of men sent down from heaven.  
Only do thou, at the boy's birth in whom  
The iron shall cease, the golden race arise,  
Befriend him, chaste Lucina; 'tis thine own  
Apollo reigns. And in thy consulate,  
This glorious age, O Pollio, shall begin,  
And the months enter on their mighty march.  
Under thy guidance, whatso tracks remain  
Of our old wickedness, once done away,  
Shall free the earth from never-ceasing fear.  
He shall receive the life of gods, and see  
Heroes with gods commingling, and himself  
Be seen of them, and with his father's worth  
Reign o'er a world at peace. For thee, O boy,  
First shall the earth, untilled, pour freely forth  
Her childish gifts, the gadding ivy-spray  
With foxglove and Egyptian bean-flower mixed,  
And laughing-eyed acanthus. Of themselves,  
Untended, will the she-goats then bring home  
Their udders swollen with milk, while flocks afield  
Shall of the monstrous lion have no fear.  
Thy very cradle shall pour forth for thee  
Caressing flowers. The serpent too shall die,  
Die shall the treacherous poison-plant, and far  
And wide Assyrian spices spring. But soon  
As thou hast skill to read of heroes' fame,  
And of thy father's deeds, and inly learn  
What virtue is, the plain by slow degrees  
With waving corn-crops shall to golden grow,  
From the wild briar shall hang the blushing grape,  
And stubborn oaks sweat honey-dew. Nathless  
Yet shall there lurk within of ancient wrong  
Some traces, bidding tempt the deep with ships,



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Gird towns with walls, with furrows cleave the earth.  
    Therewith a second Tiphys shall there be,  
    Her hero-freight a second Argo bear;  
    New wars too shall arise, and once again  
    Some great Achilles to some Troy be sent.  
Then, when the mellowing years have made thee man,  
    No more shall mariner sail, nor pine-tree bark  
    Ply traffic on the sea, but every land  
    Shall all things bear alike: the glebe no more  
    Shall feel the harrow's grip, nor vine the hook;  
The sturdy ploughman shall loose yoke from steer,  
    Nor wool with varying colours learn to lie;  
    But in the meadows shall the ram himself,  
    Now with soft flush of purple, now with tint  
    Of yellow saffron, teach his fleece to shine.  
While clothed in natural scarlet graze the lambs.  
    "Such still, such ages weave ye, as ye run,"  
    Sang to their spindles the consenting Fates  
    By Destiny's unalterable decree.  
Assume thy greatness, for the time draws nigh,  
    Dear child of gods, great progeny of Jove!  
    See how it totters- the world's orb'd might,  
    Earth, and wide ocean, and the vault profound,  
    All, see, enraptured of the coming time!  
Ah! might such length of days to me be given,  
    And breath suffice me to rehearse thy deeds,  
Nor Thracian Orpheus should out-sing me then,  
    Nor Linus, though his mother this, and that  
    His sire should aid- Orpheus Calliope,  
    And Linus fair Apollo. Nay, though Pan,  
    With Arcady for judge, my claim contest,  
    With Arcady for judge great Pan himself  
Should own him foiled, and from the field retire.  
    Begin to greet thy mother with a smile,  
    O baby-boy! ten months of weariness  
    For thee she bore: O baby-boy, begin!  
For him, on whom his parents have not smiled,  
    Gods deem not worthy of their board or bed.

## Appendix Five

### Translation of Ovid's *Heroides Epistula VII: Dido to Aeneas*

#### **Dido to Aeneas (James M. Hunter)**

Thus, when the fates call, throwing himself down in the moist grasses  
In the shallows of Maeander, sings the white swan.

*Not because I hope that you can be moved by my prayers  
Do I speak--I speak them with the gods against me;  
5 But having lost, miserably, my merit and reputation, my virtue  
Of body and spirit, to lose words is a small thing.*

Are you determined, nevertheless, to go and to abandon miserable Dido,  
And will the same winds bear away your sails and your faith?  
*Are you determined, Aeneas, to release your promises with your ships,*  
**10** And to pursue the kingdoms of Italy, which lie you know not where?  
And does not this new Carthage nor her rising walls  
Touch you, nor the high dominion given over to you?  
You flee what is done--you pursue what is yet to be done;  
You have sought one land, but must seek another through the world.  
**15** But if you find this land, who will give it for you to have?  
Who will give his fields to a stranger to hold?  
Of course another love may await you, and another Dido,  
Whom you may betray again, having given another promise.  
When will it be that you found a city as great as Carthage,  
**20** And from the high citadel look down on your people?  
If it should all happen, and there be no delay for your prayers,  
Where will there be a wife for you who loves you so?

I burn, like waxen torches covered with sulfur,  
Like pious incense placed upon the smoking hearth.  
**25** Aeneas always clings to my wakeful eyes;  
Aeneas is in my heart in the stillness and the night.  
Indeed he is ungrateful, and spurns my gifts,  
And were I not foolish, I should wish to lose him.  
Nevertheless I do not hate Aeneas, however ill he thinks of me,  
**30** But complain of his infidelity, and with the complaint I love more bitterly.  
Venus, spare your daughter-in-law; brother Love, embrace  
Your hard-hearted brother, let him serve in your camp.  
Or let me, who began it (and I am not ashamed of it), supply the love,  
While he supplies the matter for my care.

**35** *I am deluded, and this is a delusion that flies before me;  
His nature is opposed to his mother's.  
Of rocks and mountains were you born, and of the oak  
On the high cliff; you were born of savage beasts,  
Or of the sea--like the sea you see now churned up by winds,*  
**40** Across which you prepare to venture, despite the opposing waves.  
Where are you fleeing? The storm opposes you. Let the storm be my benefactor.

---

Look how Eurus stirs up the churning waters!  
What I would prefer to owe to you, let me owe to the storm.  
The wind and waves are more just than your spirit.

**45** I am not worth enough--why do I not judge *you* harshly?--  
For you to perish fleeing from me across the long waves.  
You pursue a costly hatred and purpose  
If, to be rid of me, you count it cheap to die.  
Soon the winds will calm, and with the waves spreading smoothly

**50** Triton will drive his azure steeds across the sea.  
Would that you too were changeable with the winds--  
And, unless you exceed the oak in hardness, you will be.  
Why, as if you did not know what the raging waters can do,  
Do you trust the waves whose hardships you have know so often?

**55** Even if you cast off your moorings when the sea invites the journey,  
Nevertheless the broad depths hold many woes.

Nor is it good for those who break promises to tempt the waves:  
That place exacts penalties for treachery,  
Especially when love has been wounded, for the mother of Loves,

**60** It is said, arose naked from the waves of Cytherea.

Destroyed, I fear lest I destroy; wounded, I fear lest I wound--  
Lest my enemy, shipwrecked, drink the waters of the sea.  
*Live, I pray you. Thus I shall destroy you more fully than by death.*  
*Rather, you shall be said to be the cause of my death.*

**65** Consider that you are seized--may there be no weight to the omen--  
*By a fierce storm; what will be in your mind?*

*Immediately will come the perjury of your false tongue,*

*And Dido driven to die by Phrygian deception;*

*Before your eyes will stand the face of your deceived wife,*

**70** *Sad and bloody, with streaming hair:*

*How much is it worth that then you will say "I deserve this! Pardon me!"*

*When you think that whatever thunderbolts fall were sent at you?*

Give a short space to the savagery of the sea, and your own;  
A safe voyage will be a great reward for the delay.

**75** And though you care little for this, spare the boy Iulus.

It is enough for you to have the honor of my death.

What has the boy Ascanius, what have your Penates done to deserve this fate?

Snatched from the fire, are they to be drowned in the waves?

But you are not taking them with you nor, as you claimed to me, false one

**80** Did your gods or your father ever rest on your shoulders.

You lie about everything, nor did your tongue begin to deceive

With me, nor was I the first to suffer.

If you ask, where is the mother of lovely Iulus--

She died, left alone by her harsh husband.

**85** This you told me--it was enough to warn me. I deserve

To burn; the punishment will be less than my crime.

And I do not doubt that your gods condemn you, too.

Over sea, over land, you are tossed for the seventh winter.  
When you were cast up by the waves, I received you in a safe abode,  
**90** And hardly having heard your name, I gave you my throne.  
But would that I had been content with these courtesies,  
And the tale of our common bed were buried.  
That day ruined me, when a sudden rain from the blue heavens  
Drove us into the shelter of the cave.  
**95** I heard a voice; I thought the nymphs were crying--  
It was the Eumenides giving warning of my fate.

Exact the penalty, O wounded purity, injured Sychaeus,  
To which, wretched soul, I go full of shame.  
In a marble shrine there is an image of Sychaeus, sacred to me--  
**100** Covered with leafy branches placed against it, and white fleeces.  
Thence I have heard myself called four times by a familiar voice;  
He himself in a faint voice called "Elissa, come!"

No more delay--I come, I come to you, thy rightful bride;  
I am late, however, because of my admitted shame.  
**105** Give pardon for my fault! A worthy agent beguiled me;  
He draws off the odium from my offense.  
His divine mother and aged father, burden of a dutiful son,  
Gave me hope that he would remain my rightful husband.  
If I have erred, that error had an honourable cause;  
**110** If he were to keep faith, there would be no cause for regret.  
The course of fate which was mine before still follows me  
In these last days of my life, and will endure to the end.  
*My husband was killed, struck down at the altars in his house,  
And my brother has the reward of this great crime;*  
**115** *I am driven into exile, leaving behind my husband's ashes and my homeland,  
And I flee over uncertain roads, pursued by my enemy.  
I land on this coast, having escaped my brother and the sea;  
I purchase this shore, traitor, which I gave to you.  
I found a city, and set down extensive walls*  
**120** *Arousing jealousy in neighboring kingdoms.  
Wars rumble; a woman and a foreigner, I am assailed by wars;  
I barely prepare rough gates for the city and get weapons ready.  
I am wooed by a thousand suitors, who join in complaining  
That I preferred some stranger to their marriage beds.*  
**125** Why do you hesitate to deliver me, bound, to Gaetolian Iarbas?  
I would hold out my arms for your evil deed.  
There is my brother, too, whose impious hand asks  
To be sprinkled with my blood, as with my husband's.  
Put down the gods and those sacred things which your touch profanes!  
**130** It is not good for an impious hand to honor the gods.  
If you were to be a worshipper of gods who escaped from the fires,  
Then the gods regret that they escaped the fires.

*Perhaps also it is a pregnant Dido, evil one, whom you abandon,  
And a part of me lies hidden in my body.*

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*140 The wretched infant will join the fate of the mother,  
And you will be the author of the death of your unborn child.  
With his mother will the brother of Iulus die,  
And one punishment will take us both away.*

“But your god orders you to go.” I wish that he had forbidden you to come;  
**145** Punic soil would never have felt the weight of Teucrians!  
Is this truly the god under whose guidance you are driven about by hostile winds,  
And are worn out for so long on the savage seas?  
You would hardly have such labor in returning to Pergamum,  
If Pergamum were what it had been while Hector lived.  
**150** You do not seek the Simois of your fathers, but Tiber’s waves--  
But surely, should you arrive at the place you seek, you will be a stranger;  
And this secret place so hides and so avoids your keels,  
That you will scarcely reach it in old age.

Instead, take these peoples as dowry, having given up your wandering,  
**155** And take also the wealth of Pygmalion which I carried away.  
Convey Ilion to the Tyrian city, with better fortune,  
And hold the state of king and the sacred sceptre.  
If your mind is eager for war, if Iulus seeks  
A place for his warlike spirit and for triumphs,  
**160** We shall supply enemies to conquer, and nothing shall be lacking:  
Here is a place for the laws of peace, and a place for arms.  
You must only, I pray by your mother and the weapons of your brother, the arrows,  
And by the gods, sacred to Dardanus, who are your companions in flight--  
So may they succeed, those of your people whom savage Mars  
**165** Has let escape, so may this be the limit of their loss,  
And so may Ascanius happily fill out his years,  
And the bones of old Anchises rest gently--  
You must only spare the house which has given itself to you.  
What do you say is my crime, except to love?  
**170** I am not from Phthia, or born of great Mycenae,  
Nor have my husband and father stood against you.  
*If you are ashamed of me as a wife, then let me be called not bride but hostess;  
So long as she is yours, Dido will be what you wish.*

Well known to me are the pounding seas of the African shores;  
**175** At certain times they give and deny passage.  
When the breeze gives passage, give your sails to the winds;  
Now the light seaweed holds your beached ship.  
Trust me to watch the weather; you will go more safely,  
And I myself, even though you wish it, will not let you stay.  
**180** Your comrades also ask for rest, and your mangled fleet,  
Half-repaired, demands a short delay.  
By your services, and that additional debt I may owe you,  
By my hope of marriage, I ask for a little time--  
While the seas and my love grow calm, while through time and experience  
**185** I learn to be able to endure my sorrows bravely.

If you say no, I am resolved to spill out my life;  
You cannot be cruel to me any longer.  
If only you could see the face of the one who writes these words!  
I write, and the Trojan sword is here in my lap.  
**190** Over my cheeks the tears run, onto the drawn sword,  
Which soon will be stained with blood rather than tears.  
How well your gift suits my fate!  
You furnish my grave at small expense.  
Nor is my breast now struck for the first time by a weapon;  
**195** That place has the wound of fierce love.

Anna my sister, my sister Anna, bitterly aware of my sin,  
Soon you will give the last gift to my ashes.  
Nor, consumed by the pyre, shall I be inscribed "Elissa, wife of Sychaeus";  
Just this much verse shall be on the marble of the tomb:

**200** "Aeneas provided both the cause of death and the sword;  
Dido herself struck the blow with her own hand."

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(Endnotes)

- 1 Virgil, *Aeneid*, 4:651-654, trans. John Dryden. For translations of the passages immediately surrounding the *dulces exuviae* text, see Appendix One.
- 2 A form of short unaccompanied choral composition...in use from 13th to early 16th cents. In 13th, 14th, and 15th cents. the motet was exclusively sacred and was based on a pre-existing melody and set of words to which other melodies and words were added in counterpoint. Oxford.
- 3 See appendix two for a listing of motet settings of the *Dulces exuviae*
- 4 Edgeworth quotes directly from Horace White's eloquent translation of Appian.: Appian VIII.xix. 13 1 , trans. Horace White, I (London 1912 [Loeb Classical Library]) 635-637
- 5 Edgeworth draws a symbolic connection between the fiery deaths of the two queens: "The detail of the blazing pyre, which seemed to serve no purpose in the poem, has been added in order to suggest the blazing fall of Carthage."
- 6 See Appendix Four for a full translation of *Heroides VII* by James M. Hunter.
- 7 Phrygia was a kingdom in ancient Anatolia, and is used as a descriptive epithet for Aeneas.
- 8 Dactylic hexameter is a metrical scheme utilized in Greek and Roman epic poetry. It consists of lines with six metrical feet broken up into dactyls (a long syllable followed by either another long syllable or two short ones).
- 9 See appendix two for a listing of all the occurrences of the *Dulces Exuviae* text.
- 10 Any melodic or harmonic progression which has come to possess a conventional association with the ending of a comp., a section, or a phrase. Oxford.
- 11 Included as Appendix Three. All references to measure numbers are to this edition.
- 12 Specifically in Mabriano de Orto and an Anonymous setting from the Bernard Thomas edition
- 13 A group of more than five or six notes sung to a single syllable. Oxford.
- 14 Simultaneously sounding musical lines according to a system of rules. Oxford.
- 15 An Eclogue is a lyrical poetic form which typically contains pastoral imagery.
- 16 When Constantine the Great in 324 A.D. publicly declared the establishment of Christianity as the religion of the Empire, he quoted the Fourth Eclogue as an important testimony to the recognition of the new faith by Rome's greatest poet. This gave Virgil a posthumous reputation as a sort of potential Christian before his time, to be classed in a way with the great Hebrew prophets, though somehow possessing a fuller understanding of the Christian spirit than any of them. Townend, 71,
- 17 See Appendix Three for a full translation of the Eclogue by J.W. MacHail
- 18 Schmalfeldt led the way to the original quotation: Perhaps the most notable example is to be found in the Confessions of St. Augustine (354-430 A.D.), where the early Church father looks back on his school days in Carthage and reproaches himself for having wept then over the death of Virgil's Dido when he should have been weeping over his own youthful alienation from God.
- 19 Or, in Schmalfeldt's opinion on page 588, Dido is not a mirror but a foil for Aeneas. She gives a chart that highlights the pair's opposition. For example, whereas Aeneas is "driven by duty and honor-as public, supra-personal virtues", Dido is "driven by love-a private, individual emotion, thus, potentially subversive and socially disruptive". Or, Aeneas is "rational and strives for order" while Dido "becomes irrational and creates chaos".



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# EFFECTS OF SIMULATED DEER BROWSING INTENSITY AND FRAGMENTATION ON REGENERATIVE DYNAMICS OF *ACER SACCHARUM* MARSH. (SUGAR MAPLE) PLANTS IN TEMPERATE FORESTS

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## ABSTRACT

The deer browsing effects on the regeneration of woody plants in forested ecosystems is not fully understood. More can be learned by clipping plants in specific ways to mimic some aspect of a deer browsing regimen, especially intensity. Additionally, browsing effects are not understood in the context of fragmented forests. A fragmented forest has a "forest interior" and a "forest edge", which is the area closer to the forest edge. In this study, we simulated low and high levels of natural deer browsing intensity via "clipping" of *Acer saccharum* Marsh. (sugar maple) in three fragmented forests throughout northeastern Ohio, USA. At each site, 15 plants were randomly selected in forested habitats within 40 m (forest edge) as well as 40-100 m (forest interior) from the forest edge. In each habitat, leaves of plants were either (1) clipped at high intensity (0.9 g of biomass), (2) low intensity (0.3 g), or (3) no intensity (i.e. no clipping). Plant height, aerial crown cover, and basal diameter as well as numerous branch characteristics (length, diameter, leaf and bud production) were measured before and over two years following clipping treatment. The results showed that the simulated deer browsing intensities did not influence the response variables. They also showed that leaf and bud production as well as total volume of plants were greater in forest edge habitats than in forest interiors. Our results suggest that natural levels of deer browsing intensity do not interfere with growth dynamics of juvenile sugar maple plants and that the forest edge has a higher rate of growth than forest interiors despite the old age (~ 100 years) of forest edges.

## INTRODUCTION

In forested ecosystems throughout the eastern United States, white-tailed deer (*Odocoileus virginianus* Zimmerman) is a common predator of understory woody plant species (Johnson et al. 1995). Over the last 100 years, some of these forests have experienced dramatic increases in deer densities as a result of altered hunting regulations and anthropogenic removal of deer predators (Russell et al. 2001). Increasing deer densities have led to concerns about over-browsing of woody plant species (Russell et al. 2001). Excessive browsing of juvenile woody plants, in particular, can prevent young canopy trees from transitioning from smaller to larger size classes to complete their natural life histories (Harmer 2001).

The deer browsing effects on the regeneration of woody plants in forested ecosystems is not fully understood. More can be learned by clipping plants in specific ways to mimic some aspect of a deer browsing regimen, especially intensity. (Danell et al. 1994). However, most simulated deer clipping experiments are not realistic because deer browsing regimens were not accurately characterized. One such characteristic of the browsing regimen commonly misunderstood is browsing intensity. Other studies have used low levels of simulated browsing intensity (25-30% removal of green biomass via clipping) to increase plant growth (Bergstrom and Danell 1987) and higher levels (75-100%), in contrast, to slow growth and lower survivorship (Puetmann and Saunders 2001). However, this use of percentages rarely reflects natural levels of deer browsing intensity. Also, these studies are not conducted in natural settings, so interactive effects involving browsing and other environmental factors are not implemented.

One such environmental factor is fragmentation. Forest fragmentation, due to anthropogenic disturbance, has increased the total area of forest habitats with forest edges over 100 years of age (Chen et al. 1992, Saunders et al. 1991). Compared to forest interiors, forest edges can have different levels of resources (e.g., light) (Euskirchen et al. 2000) altering plant growth and regeneration (Matlack 1994, Murcia 1995). How interactive effects like deer browsing intensity and forest fragmentation influence the regenerative dynamics of young woody plants has been poorly understood.

Our objective was to evaluate the effects of deer browsing intensity on juvenile plant growth, using *Acer saccharum* Marsh. (sugar maple) in fragmented forests of northeastern Ohio. We administered three different intensities of deer browsing via clipping, and evaluated plant growth patterns over 3 years. We sought to answer two questions: (1) Do juvenile sugar maples respond differently to deer browsing at various intensities? (2) If so, do these responses differ between juvenile sugar maples located in forest edge habitats versus forest interiors?

## METHODS

### *Study Sites*

Our study was conducted at three different sites near Cleveland, Ohio. Both Bole and Firman Woods are located at The Holden Arboretum, Geauga and Lake Counties (41°36'N, 81°18'W; 8645 ha; 244 m above sea level), and

the third site is located at Furnace Run Metro Park (41°15' N, 81°36' W; 360 ha; 340 m above sea level), which is part of the Summit County Metro Parks System. Regional temperature averages 10.8 °C, and mean monthly maximum and minimum temperatures occur in July (23.4 °C) and January (-2.7 °C), respectively (1971-2000; NCDC 2004). Annual rainfall is 91.6 cm on average, of which 60% occurs during the growing season from April through September.

The three forested sites are situated on the Glaciated Allegheny Plateau of the Beech-Maple Forest Region (Braun 1950). They are second-growth temperate forests with a canopy composed of mainly sugar maple along with *Fagus grandifolia* Ehrh., *Fraxinus americana* L., *Carya Spp.*, *Prunus serotina* Ehrh., and *Acer rubrum* L. In the understory, sugar maple, *Fraxinus americana* L., and *Lindera benzoin* Blume. predominate.

Our sites have straight, north-facing edges that are  $\geq 100$  m long. Furnace Run (Summit County, 41°15' N, 81°37' W; 156 ha) is located along a residential road, Bole Woods (Gauga County; 40° 57' N, 82° 28' W; 200 ha) is situated next to an abandoned agricultural field, and Firman Woods (Lake County; 41° 37' N, 82° 18' W; 170 ha) is adjacent to a privately owned and maintained field. Aerial photographs suggest these edges are ~100 years old.

All three forests are on gentle (2-6%) to moderately steep (6-15%) slopes and have deep soils (depth of 105-150 cm) and acidic subsoils (Williams and McCleary 1982, Ritchie and Reeder 1991, USDA 2008). The poorly drained soils that characterize Bole Woods are mostly Plateau silt loams on medium textured glacial till with a silt loam surface and a silty clay loam substratum (Williams and McCleary 1982). They also contain a fragipan layer where the water table above is seasonally perched. The moderately drained soils at Firman Woods have a seasonally high water table, but are Ellsworth silt loams with a shale substratum (Ritchie and Reeder 1991). In contrast, the soils in Furnace Run overlay a silty clay loam substratum and are characterized as a Mahoning silt loam formed from glacial till (USDA 2008).

### *Data Collection*

Data were collected over three years (2006-2008) from individually tagged sugar maple plants. In June and July of 2006, 30 juvenile plants between 30 cm and 1 m tall were randomly selected at each of the three forest habitat sites.

15 plants were selected < 40 m from the forest edge, and 15 were selected in the forest interior (40-100 m from an edge). Following plant selection, pre-clipping data were collected. Each plant was measured for height and basal diameter of the main stem. We then measured each branch off the main stem for length, diameter (at each end of a branch), as well as the number of buds and leaves. Additionally, each branch was marked with a uniquely colored piece of phone wire to keep track of the changes in these characteristics for each branch over time. A branch was defined as any living woody stem > 2 cm in length or any woody stem < 2 cm but with a living woody sub-branch > 2 cm long. Branch diameter was measured using digital calipers (accurate to  $\pm 0.01$  mm; Mitutoyo Series 500, Kanagawa, Japan).

Browsing intensity via clipping was administered in July 2006. In each forest habitat, terminal branches from each plant were randomly selected and received either one of three intensity clippings: (1) low-intensity involving the removal of 2-4 leaves ( $\sim 0.3$  g dry biomass) from a branch, (2) high-intensity or the removal of  $\sim 0.3$  g of dry biomass from each of three branches ( $\sim 0.9$  g in total), or (3) no clipping.

These clipping intensities differ from most simulated browsing studies because we did not defoliate our plants based on percentages. Instead, we simulated natural levels of deer browsing intensity. In other simulated browsing studies, researchers would remove a percentage of green biomass, ranging from 25 to 50% for low intensity (Cooper et al. 2003, Saunders and Puettmann 1999b) and 75 to 100% for high intensity (Canham et al. 1994, Saunders and Puettmann 1999a). These percentages work well for understanding plant responses to defoliation and heavy browsing. However, deers do not prefer sugar maple (Strole and Anderson 1992) and generally would not completely defoliate them. Thus, we clipped at intensities based on a typical bite size of deer (.3 g per bite) and the number of bites expected per deer browsing event (1-3 bites on average) (Shiple and Spalinger 1995).

Following clipping, each plant was fenced to prevent natural deer browsing over the course of the study. Cylindrical-shaped fences were made of 2.54 cm poultry cloth and were 2 m tall and 1 m in diameter. Plants were re-measured in July 2007 and 2008.

### Statistical Analysis

Data were analyzed in the context of a randomized block, multi-factorial experimental design with repeated measures and sampling, with simulated deer browsing intensity, location with respect to a forest edge, and time as fixed factors. At the plant level, we evaluated the effect of clipping on the collective response of unclipped branches of each plant. Response variables included total plant volume (calculated by summing all branch volumes), height to basal diameter ratio, and total number of leaves, buds, and branches per plant. Branch volume was calculated as the volume of a frustum using the following equation,

$$V = \frac{p * length}{3} (BD_{bot}^2 + BD_{bot} * BD_{top} + BD_{top}^2)$$

These data were analyzed using analysis of covariance (ANCOVA) with pre-treatment data used as the covariate.

At the branch level, we examined mean branch volume and mean branch length involving all branches. These data was analyzed using ANOVA. Also, we compared those branches which were clipped in 2006 to randomly selected terminal branches from unclipped control plants and examined changes in the number of leaves and buds per branch. Branches that died in 2007 and 2008 were excluded from these analyses, and data were analyzed using ANCOVA. Analyses were performed using PROC MIXED in SAS 9.1 for Windows (SAS, 2001). When necessary, data were natural-log transformed to meet model assumptions; for all analyses,  $\alpha = 0.05$ .

## RESULTS

### Plant level responses

At the plant level, the separate and interactive effects of deer browsing intensity and fragmentation were generally not detected (Table 1). However, plant volume was affected by an interaction involving fragmentation and time ( $p = 0.049$ ). In 2008, plant volume in edge habitats was 32% greater than that in forest interiors (Tukey-Kramer  $p = 0.007$ , Figure 1, next page). Additionally, while an interactive effect of time, fragmentation, and browsing intensity on the number of branches per plant was detected ( $p = 0.021$ ), this was attributed to changes over time in edge habitats receiving low clipping and interior habitats receiving high clipping (Tukey-Kramer  $p = 0.003$  and  $p = 0.012$  respectively) as well as a difference in edge habitats

receiving low clipping in 2007 versus interior habitats receiving high clipping in 2008 (Tukey-Kramer  $p = 0.050$ ).

#### *Branch level responses*

Fragmentation, alone, affected branch level responses of juvenile plants (Table 2). Specifically, fragmentation influenced bud ( $p = 0.053$ ) and leaf ( $p = 0.037$ ) production. Plants in edge habitats had 45% more buds than those in forest interiors (Figure 2a). Similarly, plants near a forest edge had 53% more leaves than those located in forest interior habitats (Figure 2b).

## **DISCUSSION**

Compared to plants in forest interiors, plants had greater volume as well as more leaves and buds in forest edge habitats where light levels are likely elevated (Euskirchen et al. 2000). Since our edges were ~100 years old, these results were unexpected. Light levels in forest edge habitats usually diminish after 40-50 years; edges “close up” following adventitious lateral growth of canopy adults near such edges (Matlack 1994). In contrast, our fragmented sites likely remained open, causing plant growth and regeneration.

At our sites, a single natural deer browsing event at various intensities did not affect growth and regeneration of juvenile sugar maple plants. In contrast to our results, woody plants have demonstrated in the past overcompensation of growth after levels of simulated browsing intensity (Bergstrom and Danell 1987, Danell 1985, Hjalten et al. 1993). This response may represent an attempt to escape a ruminant browse line (Bergstrom and Danell 1987) or maintain a constant root to shoot ratio (Danell et al. 1985). Alternatively, overall plant growth can decrease

(Puetmann and Saunders 2001), possibly due to a decrease in competitive ability (Augner et al. 1997). We did not observe either of these changes perhaps because our browsing intensity treatments did not involve unnatural levels of plant defoliation using arbitrary percentages.

Regenerative dynamics of juvenile sugar maple plants may be affected by other characteristics of a deer browsing regimen, including seasonal timing, and frequency (Pickett and White 1985). Seasonal timing of browsing can affect plant responses (Hjalten et al. 1993). Plants browsed in the early summer have a greater response to clipping than those browsed in winter or late summer (Reich et al. 1993). Little knowledge about the effects of browsing frequency on plant regeneration is available because most browsing studies do not imitate natural frequencies and only clip once a year (see Bergstrom and Danell 1987). Information about the effects of seasonal timing and browsing frequency is crucial in accurately simulating browsing regimens and could help researchers better understand how natural browsing affects woody plant regeneration.

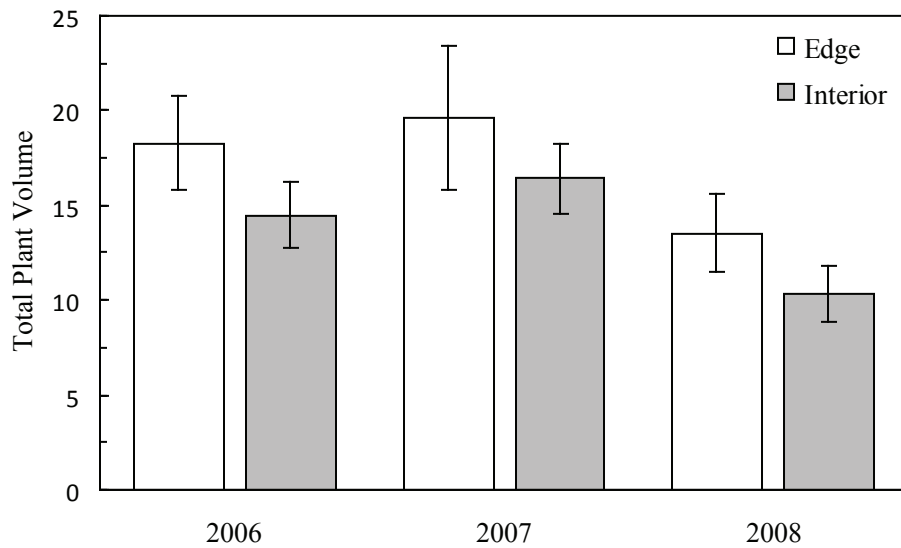
We therefore suggest two lines of research that would help elucidate the natural effects of deer browsing on woody plant regeneration. The first is to begin research projects in line with the projects of Shipley and Spalinger (1995) to gain a deeper understanding of deer browsing regimens. In order to understand deer browsing regimen, more research is needed on the frequency and seasonal timing that deers browse woody plants. After the deer browsing regimen is understood, we can more accurately research the effects of natural browsing on plants to then know how to most appropriately manage deer populations in temperate forests.

Response	Covariate	Location	Intensity	Location* Intensity	Time	Location* Time	Intensity* Time	Location* Intensity* Time
Volume	< 0.001	0.210	0.278	0.125	< 0.001	<b>0.049</b>	0.739	0.621
Height to BD ratio	< 0.001	0.773	0.236	0.873	0.740	0.291	0.374	0.874
Leaves	0.004	0.953	0.283	0.550	0.306	0.778	0.752	0.414
Buds	< 0.001	0.771	0.144	0.534	0.003	0.942	0.283	0.347
Branches	0.348	0.506	0.541	0.793	< 0.001	0.337	0.086	0.021

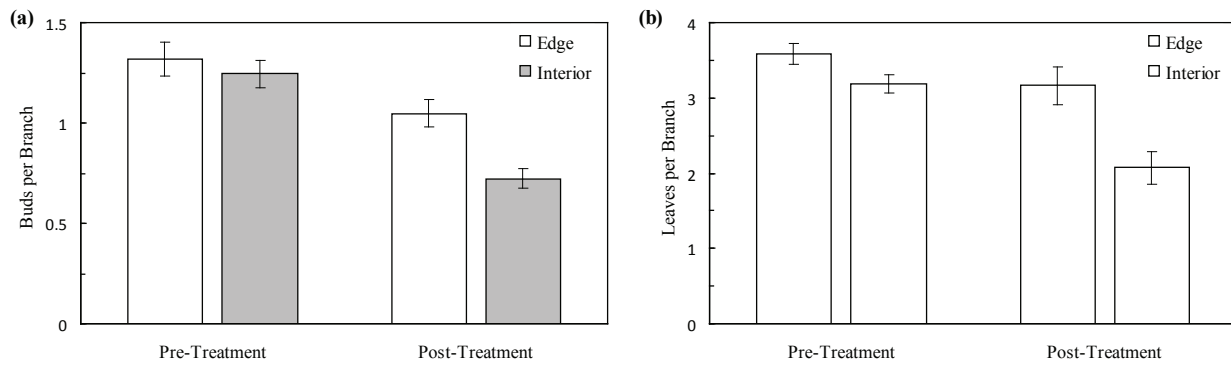
**Table 1:** Sources of variation and *P* values for fixed effects following ANCOVA that was used to examine plant level effects of simulated deer browsing intensity and fragmentation on juvenile sugar maple plants.

Response	Covariate	Location	Intensity	Location* Intensity	Time	Location* Time	Intensity* Time	Location* Intensity* Time
Mean Branch volume	--	0.952	0.254	0.454	0.369	0.263	0.998	0.362
Mean Branch length	--	0.378	0.289	0.221	0.294	0.143	0.708	0.313
Mean Buds per branch	0.020	<b>0.053</b>	0.210	0.992	0.017	0.080	0.984	0.716
Mean Leaves per branch	0.351	<b>0.037</b>	0.318	0.843	0.168	0.296	0.666	0.845

**Table 2:** Sources of variation and *P* values for fixed effects following ANCOVA that was used to examine branch level effects of simulated deer browsing intensity and fragmentation on juvenile sugar maple plants.



**Figure 1:** Mean ( $\pm$  SE) volume of juvenile sugar maple plants in forest edge and interior habitats before (2006) and after (2007, 2008) simulated deer browsing intensity.



**Figure 2:** Mean ( $\pm$  SE) number of (a) buds per branch and (b) leaves per branch for juvenile sugar maple plants in forest edge and interior habitats before (Pre-treatment) and after (Post-treatment) simulated deer browsing intensity.

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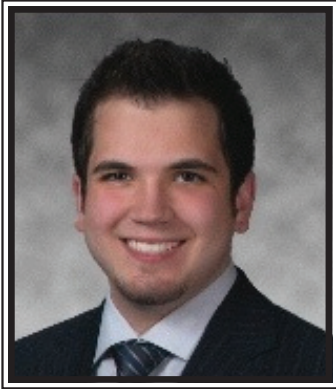


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# THE ROLE OF INNATE RECEPTOR TLR2 IN NEUTROPHIL RECRUITMENT IN OROPHARYNGEAL CANDIDIASIS (OPC)

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-Saleh Al-Kharsa-

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## -Acknowledgments-

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## ABSTRACT

*Candida albicans* (CA) is a dimorphic fungus that commonly colonizes the oral cavity. Overgrowth can result in an infection of the oral cavity, known as oral candidiasis (OPC). Neutrophils play a major role in nonspecific immune defense against fungal infection. Neutrophils then engage in phagocytosis of the *C. albicans* by engagement of membrane innate receptors, called pattern recognition receptors (PRRs) that interact with certain identifying molecules on the cell wall of the infecting fungus, called pathogen-associated molecular patterns (PAMPs). Toll Like Receptors (TLRs) are a type of PPR expressed on neutrophils and are believed to play a major role in its activation and migration.

This study evaluates the role of TLR1, 2, and 6 in neutrophil recruitment in tongues utilizing a murine model of OPC. Tongue sections from C57BL/6, TLR1, TLR2, and TLR6 KO mice were stained with a neutrophil specific NIMP/alex488 antibody. Neutrophil recruitment was quantitatively assessed by comparing the amount of fluorescence in the tongue epithelium.

Our results found that the lowest percent fluorescence was experienced in the tongue sections of the TLR2KO mice meaning that neutrophil recruitment was hindered. We also observed hindered neutrophil recruitment in the TLR1, and TLR6 KO mice, however it was not as severe as in the TLR2 KO mice. These results imply that all three TLR types 1, 2, and 6 play a role in neutrophil recruitment in the OPC model.

## INTRODUCTION

The human oral cavity serves as a reservoir for hundreds of commensal microbes that coexist within a delicate balance, between microbes and the host immune system. These microbes live together in complex networks termed biofilms, in which the microbes interact cooperatively to promote survival and growth. Many parameters affect this biofilm such as temperature, pH, oxygen, fluid flow, as well as the host's age. In particular, the tongue offers microbes an extremely fertile environment for biofilm colonization, due to its numerous protected sites<sup>1</sup>.

Any disturbance in the oral cavity's normal flora can allow for the overgrowth of normal commensal organisms, such as *Candida albicans*, resulting in a pathogenic infection. The propagation of this fungus can result

in a very painful and in some cases fatal condition known as oropharyngeal candidiasis (OPC)<sup>2</sup>. Candidiasis is an opportunistic pathogen, meaning that it usually does not cause disease in healthy individuals. Hence, hosts who are immunocompromised; typically elderly, those who suffer from AIDS, people that undergo chemotherapy, infants, or transplant patients, are at higher risk of infection. CA is the 4th most common life threatening infection among all bloodstream and nosocomial infections in the United States<sup>3</sup>.

*Candida* species are dimorphic fungi capable of existing in both a yeast form and a hyphal form. One of the complicating factors of *Candida* infection is its ability to switch morphogenic states at body temperature. Yeast and hyphae express different surface molecules at different levels making the initiation of effective immune responses to infection more difficult<sup>4</sup>. When invading tissue, *Candida* is most commonly found in the hyphal form, which is capable of destroying surrounding tissue via mechanical processes. The first line of defense against infection involves the physical barrier provided by epithelial cells. Once *Candida* has broken through the barrier of the epithelia, control of infection and prevention of dissemination involves the immediate activity of the innate immune system.

The main cells of the innate immune system that recognize invading pathogens are monocytes and neutrophils. Neutrophils are polymorphonuclear, phagocytic cells that are part of the innate immune system's first responders to a pathogen infection. Upon infection, a variety of chemical mediators, called cytokines and chemokines, are released resulting in the increased surface expression of adhesion molecules involved in neutrophil infiltration and trigger dilation in the blood vessels around the infected area. This in turn causes added permeability of blood vessels and endothelial cells. Neutrophils then migrate from capillaries and pass through the endothelial layer to the site of infection. This migration from the blood to the site of infection utilizes a process known as chemotaxis. Chemotaxis involves the movement of neutrophils from areas of low chemokine concentration, such as blood, to areas of high chemokine concentration, the site of *Candida* infection. There is a multitude of protein and carbohydrate molecules that play a critical role in the migration of neutrophils to sites of infection.

Until recently, little was known about the process by which neutrophils recognize *C. albicans* as a pathogen.

The oversimplified belief that innate immunity was non-specific and employs an 'ingest and destroy' method could not explain the specific and diverse responses of innate immunity. Current research shows that pattern recognition receptors (PRRs) assume the role of recognizing, for the innate immune system, invading pathogens including *C. albicans*. PRRs recognize conserved molecular motifs within pathogens called pathogen-associated molecular patterns (PAMPs). In mammalian molecules, four types of PRRs have been identified as Toll-like receptors (TLRs), C-type lectin-like receptors, Nucleotide-binding oligomerization domain (NOD) like receptors, and retinoic-acid-inducible gene receptors.

TLRs are transmembrane proteins containing an extracellular domain to identify PAMPs and an intracellular domain that activates signaling cascades upon PAMP interaction. Currently, 10 TLRs have been identified in humans that are capable of engaging specific PAMPs. An example is TLR2, which recognizes phospholipomannan, a phylogenetically unique fungal component. C-type lectin-like receptors mainly constitute membrane bound receptors that recognize polysaccharide structures, such as  $\beta$ -glucans, that are found in the cell wall of *C. albicans*<sup>5</sup>. Nucleotide-binding oligomerization domain (NOD)-like receptors and retinoic-acid-inducible gene receptors are both cytoplasmic receptors, involved in recognizing viral nucleic acids. No studies have documented any involvement of these receptors in the recognition of fungal pathogen components<sup>6</sup>.

Research utilizing fluorescent analysis of neutrophils stained with anti-TLR antibodies has indicated that neutrophils have been shown to express all known TLRs except for TLR3<sup>7,8</sup>. These TLRs play a role in the recognition of the components of fungal pathogens, and further activation and recruitment of neutrophils<sup>9</sup>. The aim of this research is to investigate neutrophil recruitment into the epithelial tissue of the tongue in response to oropharyngeal candidiasis (OPC) and its dependence on TLR2 and its co-receptors TLR1 and TLR6. Due to the ability of TLR2 to recognize fungal PAMPs, we expect to see a much lower neutrophil count in tongue sections of TLR2KO mice. Doing this will give us a better understanding of the specific roles of TLRs 1, 2, and 6 in the recognition of *C. albicans* for neutrophil recruitment, and further help us understand the mechanisms that propagate the innate immune response.

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## MATERIALS & METHODS

### *Animals*

C57BL/6 mice were used as the control for this experiment. They were obtained from Jackson Laboratories (Bar Harbor, ME). TLR2KO, TLR6KO, TLR1KO mice were graciously provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan). All animals used in this experiment were housed in filter-covered micro-isolator cages. During breeding and storing they were housed in the Wolstein Animal Facility at Case Western Reserve University. Mice were then transferred to the Wearn Animal Facility at University Hospitals in Cleveland for infection and the duration of the experiment. Case Western Reserve University School of Medicine Institutional Animal Care and Use Committee has approved all protocols and experimen

### *Fungal preparation*

The strain of *C. albicans* (CA) that was utilized in this study was GDH2346, originally isolated from a denture stomatitis patient. The strain was propagated and maintained in Sabourand Dextrose (SD) agar at 4°C. Prior to mouse infection *C. albicans* was scraped off the SD agar and transferred to a yeast-inducing SD broth (Difco, Sparks, MD) and incubated at 37°C with a shaking for a period of 16 – 24 hrs. After incubation, yeast was collected and washed 3x with PBS. Yeast was spun down and pelleted using a centrifuge between each wash step. The CA was then suspended in PBS and cells were counted on a hemacytometer. Yeast was then diluted to the desired concentration of 5x10<sup>7</sup> yeast/mL OPC infection Model-Five days before infection with *C. albicans*, the mice were fed tetracycline water (2.5g/L), a form of antibiotic, to reduce competing oral flora. The mice were then injected with an anesthesia cocktail (25% Ketamin (Vedco, St Joseph, MO), 7% Acepromazine (Boehringer Ingelheim, Germany)). After the mice had been properly anesthetized, 4-6 evenly spaced incisions were made on the dorsal layer of the tongue making sure not to pass the epithelium and cause bleeding. All incisions were made using a sterile #10 blade scalpel. An autoclaved cotton ball saturated with PBS was then inserted into the mouse's mouth to prevent dryness. After 3 hours the cotton ball was removed and 100 µL of 5.0 x 10<sup>7</sup> CA suspension was pipetted onto the tongue with a new cotton ball, and the animal was left for approximately 4-6 hours while sedated. After a three day infection period the mice were then euthanized us-

ing a CO<sub>2</sub> asphyxiation chamber. Using sterile autoclaved instruments the tongue was removed and treated for slide preparation. Three different BL6 mouse tongues were obtained as well as two from each of the TLR1KO and TLR6KO mice, and finally only one TLR2KO mouse tongue was obtained.

### *Immunohistochemistry*

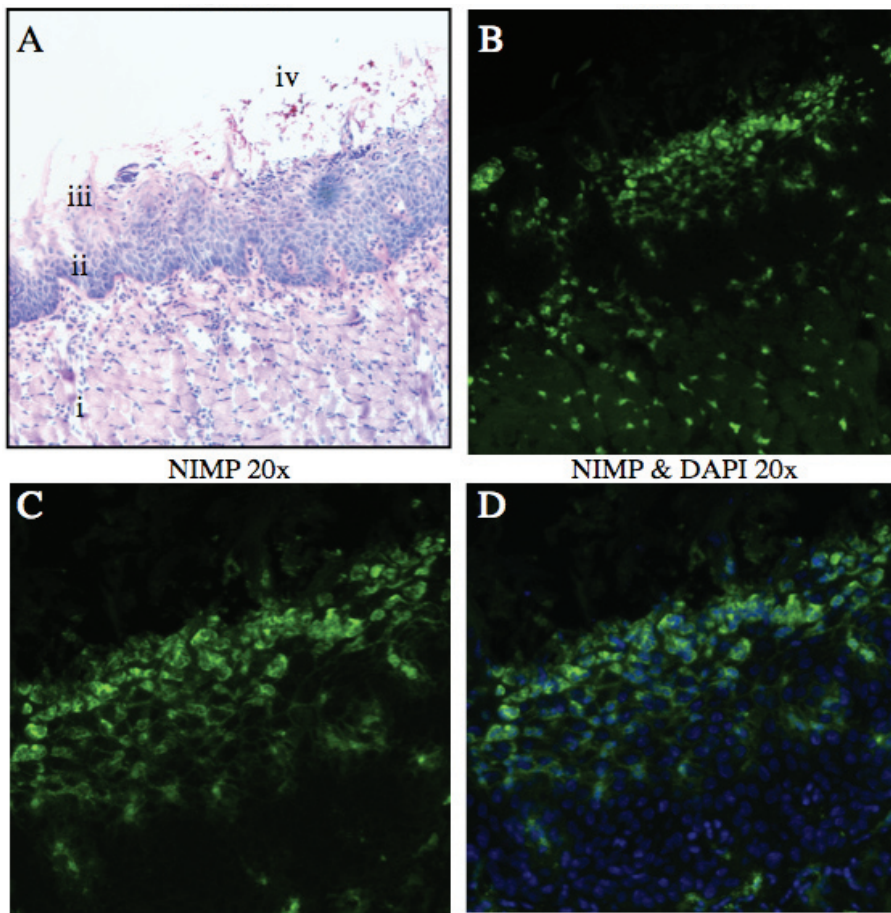
After the tongues were removed they were immersed in Embedding Media (Tissue Tek) (EMS Hatfield, PA) and flash frozen using liquid nitrogen. The tongues were then transferred on dry ice to a microtome-cryostat to be sectioned. From each tongue 2 slides containing two 5-micron sections each, and one slide containing two 10-micron sections were prepared. All slides were then stored in a -20°C refrigerator.

The 10-micron slide was stained with Periodic acid-Schiff and hematoxylin (PASH). One of the 5-micron slides was stained with an anti-neutrophil antibody (NIMP supernatant) and incubated for 2 hours. After washing with PBS-triton the slide was then stained and incubated for 45 minutes with the secondary antibody conjugated to the green fluorochrome Alexa-488 at a dilution of 1:400 in 1% normal goat serum, which provided the fluorescent tag that allowed neutrophil detection and imaging with the florescent microscope. After washing again with PBS-triton the slide was then mounted in Vectashield containing the nuclear stain DAPI (Vector Laboratories, Burlingame, CA). The other 5-micron slide was used as the negative control for the neutrophil staining. This was done by using the same method for staining the first 5-micron slide without using the primary antibody (NIMP).

### *Microcopy and Quantification*

The images were taken using a Leica DMI 6000B inverted microscope. For each NIMP slide, a 10x resolution image was taken of the whole tongue at an exposure of 4500 milliseconds. This was accomplished by stitching together a large number of consecutive 10x images of the tongue. In addition to the stitched image a 20x image was taken of the area of high fluorescence both in green fluorescence (Alex488) as well as a combined superimposed image of green and blue fluorescence (Alexa488 & DAPI).

For the PASH stained slides a 10x stitched image of the whole slide was taken as well as 20x images of the area of the epithelium where fungal hyphal infiltration was observed.



**Figure 1:** Sections of C57BL/6 OPC infected mouse tongue epithelium tissue, stained with PASH stain to show histology and *C. albicans* growth, and NIMP/Alexa488 and DAPI stain on the neighboring tongue section to show neutrophil migration and nucleus placement. A) PASH stain of tongue epithelium (blue) *C. albicans* is seen on the top of the epithelium (magenta). B) corresponding tongue section with NIMP/Alexa488 (green). Neutrophils fluoresce green and are migrating to the site of *C. albicans* infection. C) 20x image of the same section. D) Same section with NIMP/Alexa488 (green) stain and DAPI (blue) stain.

To quantify the number of neutrophils in each tongue section, the cell counting technique was opted for digital quantifying method using the imaging program Metamorph. For each image taken by the program a number between 1 and 4095 is assigned for each pixel corresponding to its “fluorescing” value. To quantify the results we used the 10x-stitched images of each tongue and using Metamorph the epithelial region of the tongue was selected. In the selected region the total amount of pixels were counted as well as the total amount of pixels that had number values that was considered fluorescing, which was set between 715 and 4095. The percent fluorescence was calculated by dividing the number of fluorescing pixels by the total amount of pixels in the epithelium.

## RESULTS & DISCUSSION

Using NIMP/Alexa488 staining, we visualized neutrophil recruitment in the tongues of different OPC infected TLRKO mice and compared them to the BL6 mouse control. In addition to qualitative assessment done with the fluorescent microscope, we used the analytical program, Metamorph. With Metamorph we quantified our results in order to better analyze the neutrophil recruitment in the tongue epithelium. Figure 1 displays PASH staining and neutrophil staining of a BL6 mouse. Image A displays the PASH stain showing the tongue histology. Part ‘i’ shows the muscle and blood vessel tissue, ‘ii’ shows the superior epithelium of the tongue, ‘iii’ shows the outer most layer of the tongue the stratum corneum that consists of keratinized oral mucosal, and finally ‘iv’ shows the *C. albicans* infiltration and growth on the surface of the tongue. Images B & C show 10x and 20x images of NIMP/Alexa488 staining of that same location on a serial section of the tongue. The green fluorescence corresponded to neutrophil specific staining. Finally, image D shows a superimposed NIMP/Alexa488 and DAPI staining. DAPI stain is a DNA specific fluorescent stain, hence the blue fluorescence in this image corresponds

to all the nuclei in the image. Looking at the neutrophil nuclei we can visualize the polymorphonuclear properties of these cells confirming that our NIMP/Alexa488 stain corresponded to neutrophils.

Figures 2, 3, 4 and 5 display PASH and NIMP/Alexa488 staining of OPC infected tongues for BL6, TLR1KO, TLR2KO, and TLR6KO mice respectfully. Qualitatively we saw a trend of a much weaker neutrophil recruitment response to the *C. albicans* infection in all of the gene knockout mice compared to the BL6 control. Also we noticed another trend where the *C. albicans* growth and epithelium infiltration were more prominent on the tongues of the TLR1KO, and TLR6KO mice.

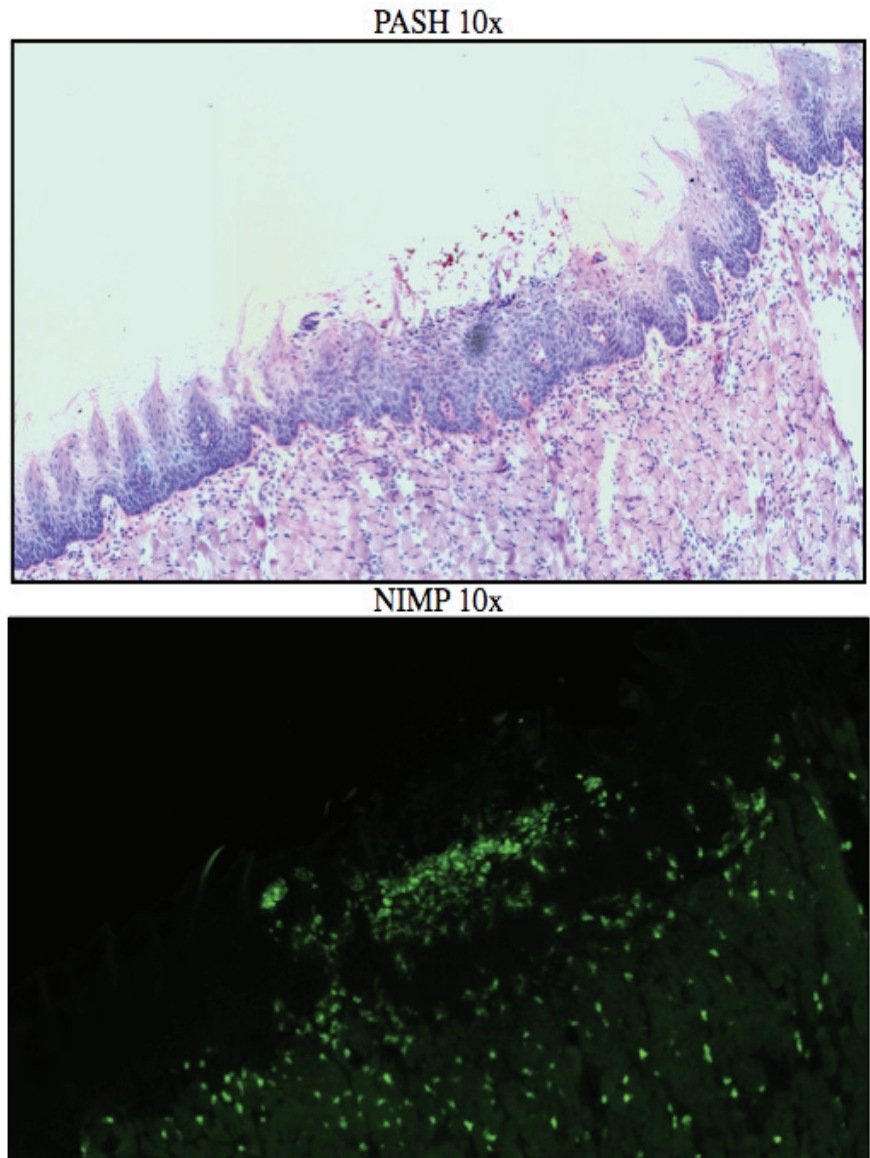
We used Metamorph to calculate the presence of fluorescence for each of the tongue sections stained. We then calculated average percent of fluorescence for each

mouse genotype and we graphed the values in Figure 6. Our results are consistent with our qualitative analysis of the staining; we saw a negative trend in neutrophil recruitment with any of the knockout mice. We saw the lowest percent of fluorescence in our TL2KO mouse. The calculated fluorescence value for the TLR2KO mouse was 0.61% compared to the average 1.7% fluorescence of the Bl6 control mice. TLR2 is a cell surface receptor present on neutrophils. It has been implicated in the recognition of the lipopeptide phospholipomannan, a component *C. albicans*. The neutrophils in TLR2KO mouse lack this surface receptor and hence the ability for them to migrate to the site of the *C. albicans* infection becomes severely hindered.

Our TLR1KO, and TLR6KO mice also had hindered neutrophil recruitment, 0.72% and 1.1% respectfully. Both TLR1 and 6 form heterodimers with TLR2 to provide it with ligand specificity. TLR2/TLR1 heterodimers recognize triacyl lipopeptides, and TLR2/TLR6 heterodimers recognize diacyl lipopeptides<sup>10</sup>. When comparing the fluorescence values of both genotypes there was less neutrophil recruitment in the TLR1KO mouse, which means that there was a larger number of triacyl lipopeptide ligands involved in the recruitment of neutrophils, however more thorough research is needed.

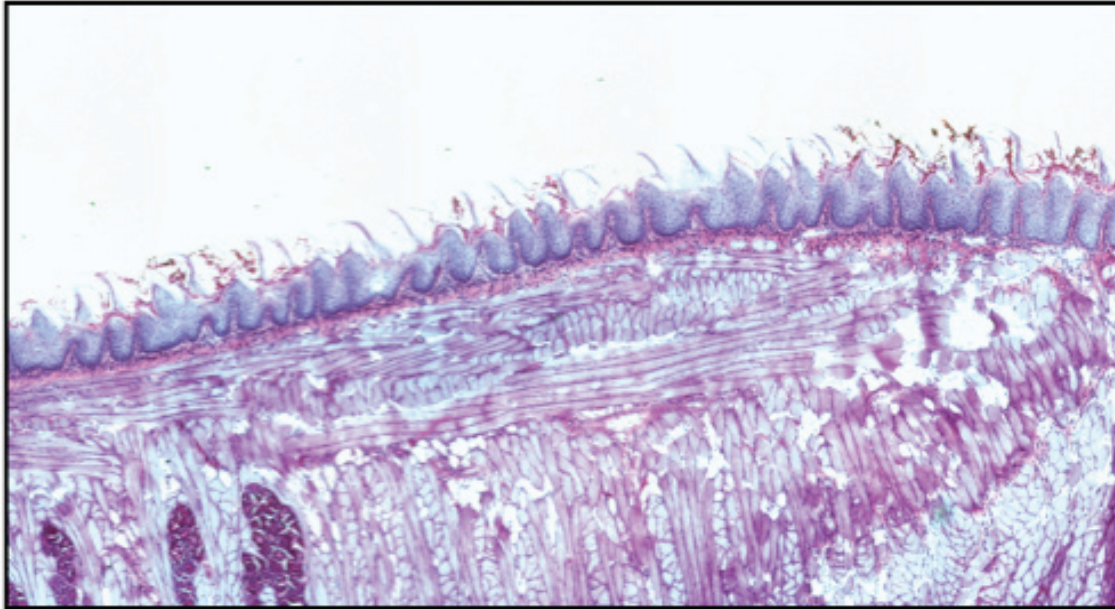
None of the gene knockout mice displayed zero fluorescence/neutrophil recruitment. This suggests that the signaling pathway for neutrophil recruitment is not solely dependent on either TLR1, 2, or 6 and that neutrophil recruitment is not dependent on one signaling pathway. In a preliminary study, we observed very little to no fluorescence in a NIMP/Alexa488 stained MyD88KO mouse tongue section. This is an indication that MyD88 might be responsible for a signaling pathway that may or may not directly involve TLRs.

In the future, studies will include a larger sample size to give more significant results. Additional studies will further characterize the role of MyD88 and C-type lectin receptor Dectin-1, a receptor believed to collaborate with TLRs<sup>11</sup>, in neutrophil recruitment to a candidiasis infection.

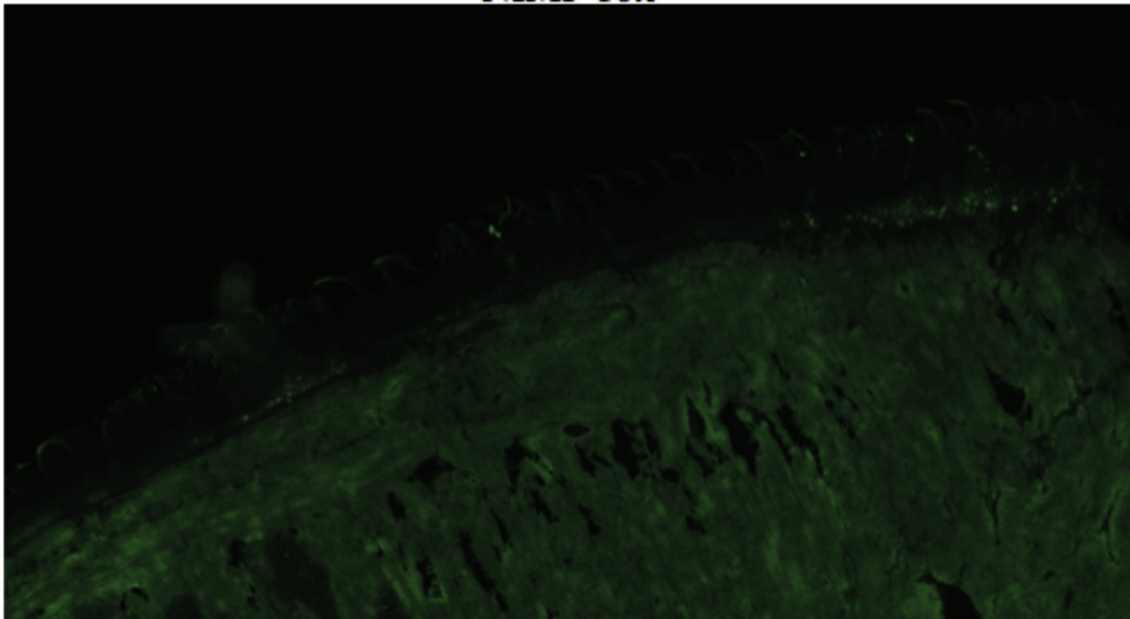


**Figure 2:** Sections of C57BL/6 OPC infected mouse tongue epithelium tissue, stained with PASH stain to show histology and *C.albicans* growth, and NIMP/Alexa488 stain on the neighboring tongue section to show neutrophil migration.

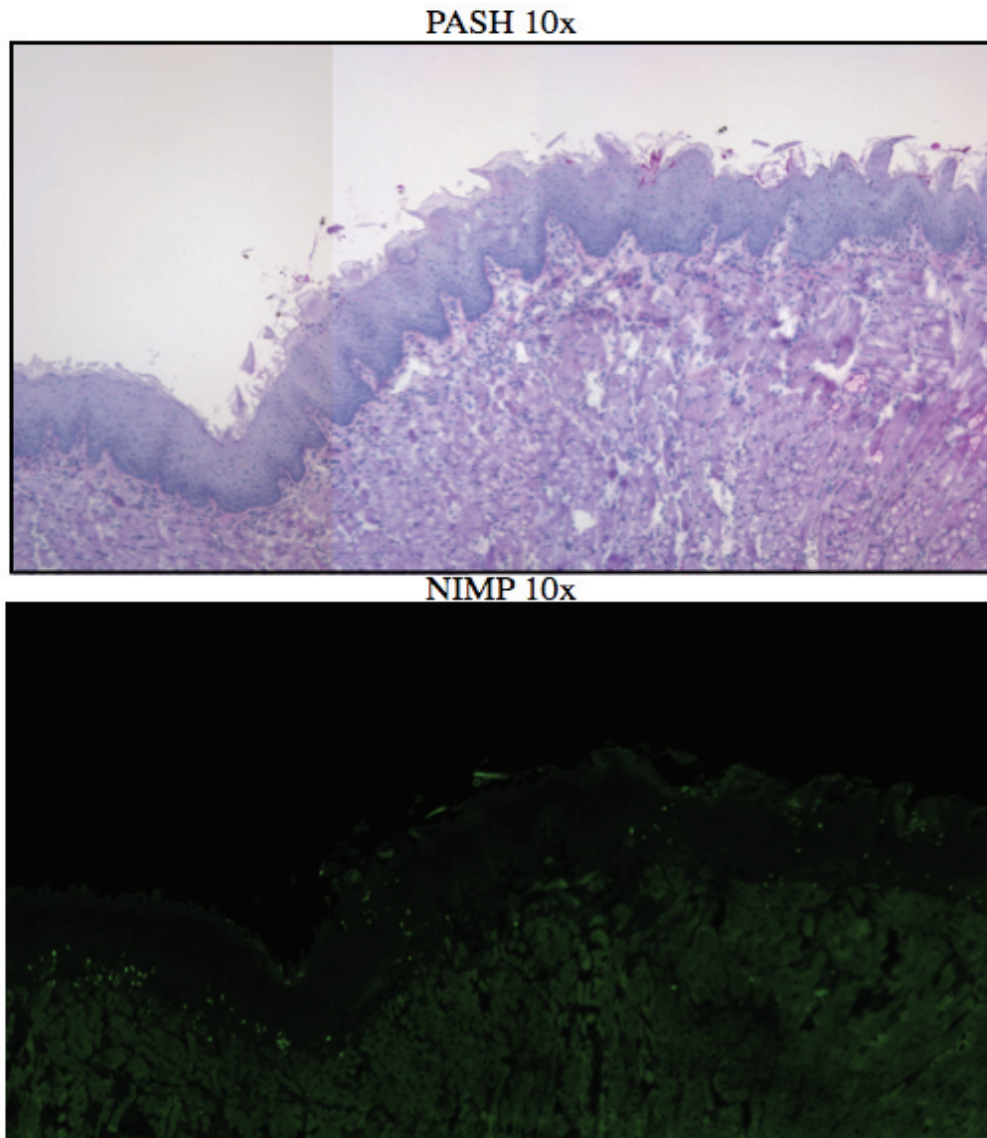
PASH 10x



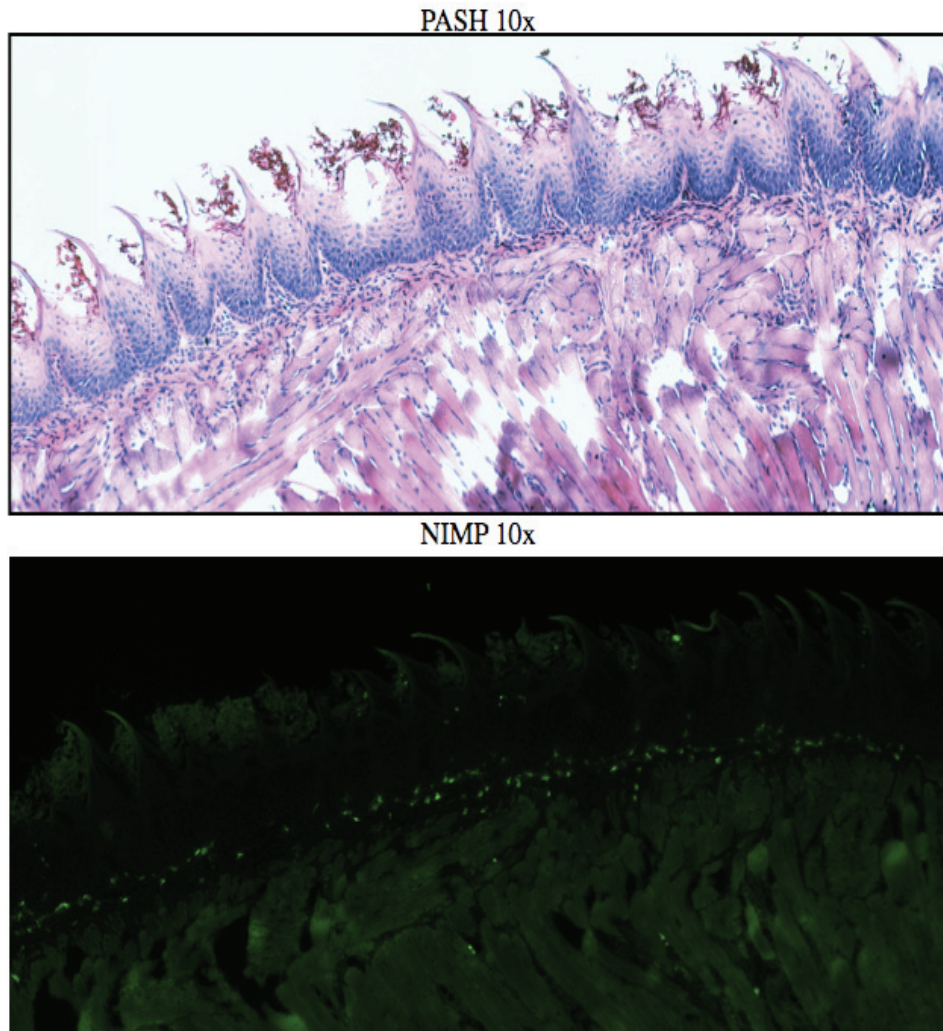
NIMP 10x



**Figure 3:** Sections of TLR1KO OPC infected mouse tongue epithelium tissue, stained with PASH stain to show histology and *C. albicans* growth, and NIMP/Alexa488 stain on the neighboring tongue section to show neutrophil migration.

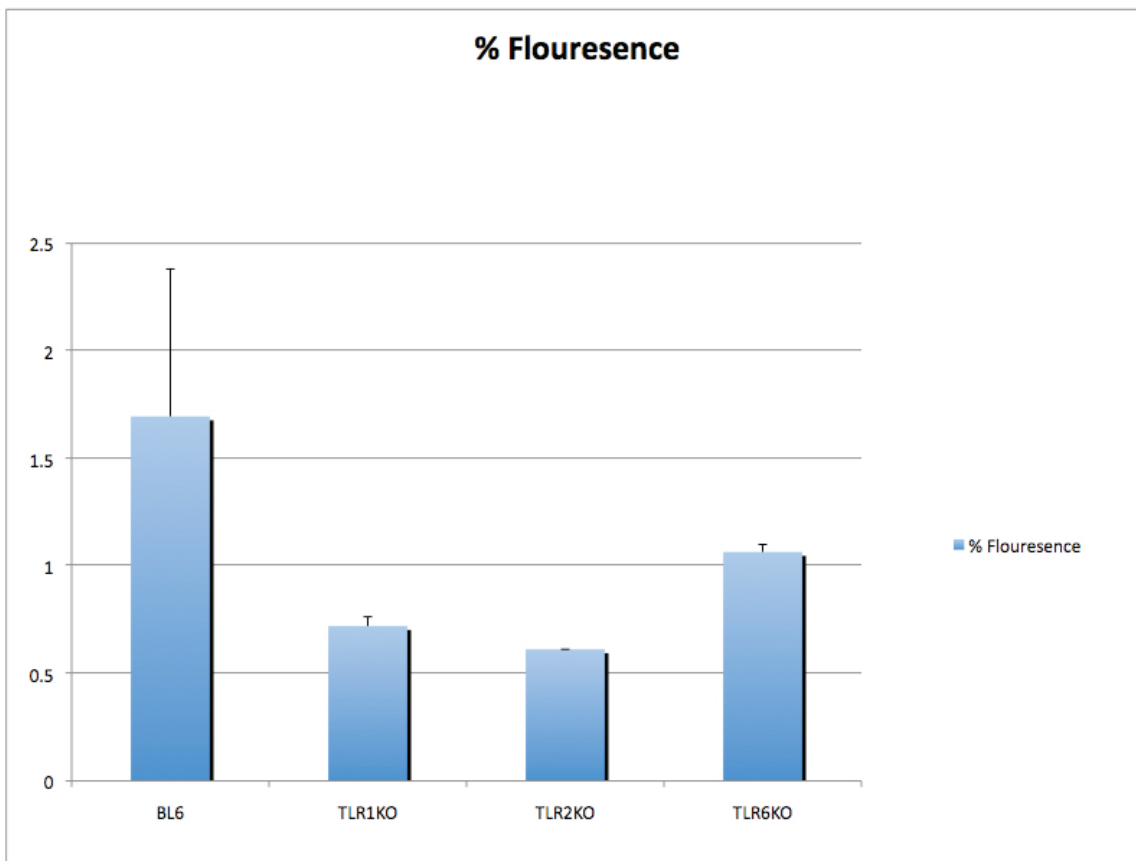


**Figure 4:** Sections of TLR2KO OPC infected mouse tongue epithelium tissue, stained with PASH stain to show histology and *C.albicans* growth, and NIMP/Alexa488 stain on the neighboring tongue section to show neutrophil migration.



**Figure 5:** Sections of TLR6KO OPC infected mouse tongue epithelium tissue, stained with PASH stain to show histology and *C.albicans* growth, and NIMP/Alexa488 stain on the neighboring tongue section to show neutrophil migration.





**Figure 6:** The average percent fluorescence for each of BL6, TLR1KO, TLR2KO, TLR6KO OPC infected mice and their corresponding sample sizes (n). The fluorescing corresponds to the neutrophils in the tongue sections from the NIMP/Alexa488 staining. The percent of fluorescence was calculated by dividing the number of fluorescing pixels by the total number of pixels in the 10x-stitched images in the program Metamorph.

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# *Media Board*



Media Board is a student group comprised of representatives from each of Case's student media organizations. It promotes responsible forums of student expression in the various media.

Member organizations include The Athenian (Humor Magazine), Case Reserve Review (Literary Magazine), Discussions (Undergraduate Research Journal), Engineering & Science Review, Film Society, Ignite TV, The Observer, Retrospect (Yearbook), and WRUW 91.1 FM.

Since these groups are all student run, they always welcome new members! Any interested students should check out the Media Board website for more information at <http://studentaffairs.case.edu/groups/mediaboard/>.

Any media oriented student group interested in becoming a member of Media Board is welcome to apply for membership and should contact the Media Board Chairman at [mediaboard-chairman@case.edu](mailto:mediaboard-chairman@case.edu).

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