CABRINI

2016 JOURNAL OF UNDERGRADUATE RESEARCH


GRACE HALL

TABLE OF CONTENTS

CONTENTS

ARTS & HUMANITIES

LIFE & PHYSICAL SCIENCES

LETTER FROM THE PRESIDENT

March 31, 2016

Cabrini College Community,

Most people are familiar with the adage that says, "Learning takes place everywhere." That's true, especially on a college campus (and particularly on a liberal arts campus).

However, some types of learning have a more profound impact on the learner, and create an indelible impression that can be transformative.

Doing undergraduate research is exactly this type of learning, and I'll wager that students who contributed to this Journal of Undergraduate Research won't soon forget the experience.

Congratulations to every student who has work published in this Journal of Undergraduate Research, and to the co-editors, editorial review board, students, faculty, and staff who produced this publication.

My hope is that every student who published research in this Journal feels pride and a sense of accomplishment because you've certainly earned the right.

Doing research like this is what makes the College so extraordinary and places Cabrini students among the best.

Again, congratulations, and enjoy showing this Journal to friends and family.

Warm regards,

Donald B. Taylor, PhD
President

EDITORIAL STATEMENT

The Cabrini College Journal of Undergraduate Research is an annual, reviewed publication dedicated to the discovery, promotion, and publication of outstanding work done annually by Cabrini undergraduates.

The Journal's Editorial Board reviews, selects and cultivates the best work for inclusion. Drawn from the Undergraduate Arts, Research, and Scholarship Symposium—an annual event where students present and showcase their research to the College community—the Board seeks academically rigorous and distinctive efforts that demonstrate Cabrini students' evolution into public intellects with a firm grasp of the stakes and conventions of meaningful scholarship.

Articles are selected for publication based on their scholarly and rhetorical quality. They are from all disciplines and exemplify one or more of the following accomplishments:

- An original research project
- Unique contribution to the scholarship of the student's field
- A new interpretation of an intellectually important problem; phenomenon or text
- An interdisciplinary endeavor that suggests an innovative approach to an altogether new subject for scholarly inquiry

The board also considers for publication any work of artistic merit that demonstrates academic seriousness and intellectual ambition.

CO-EDITORS

- John W. Cordes, PhD - Department of Communication
- David Dunbar, PhD - Department of Science

REVIEWERS

- David Dunbar, PhD - Department of Science
- Melinda Harrison Krick, PhD - Department of Science
- Caroline Nielsen, PhD - Department of Science
- Sharon Schwarze, PhD - Department of Philosophy
- Tamarah Smith, PhD - Department of Psychology
- Melissa Terlecki, PhD - Department of Psychology
- Nicholas Uliano, PhD - Department of Romance Languages and Literatures

ARTS & HUMANITIES

Bilingual Education

Author: Brittany Grenyer
Faculty Mentor: Raquel A. Green, PhD

Abstract: This paper discusses the advantages of a greater inclusion of bilingual education in the American school systems. It also addresses programs that will improve academic achievement, self-esteem and social adjustment. Finally, it includes a discussion of the author´s personal experience with bilingual education, and the impact it has had on her education, skills and awareness.

Bilingual education has been a politicized topic of debate for years. There are many who support bilingual education being incorporated into educational curriculums, while others are adamant that it should not be a part of the classroom. Those that are opposed to bilingual education seem to question the idea of students being exposed to a second language or becoming proficient in two languages. Bilingual education has many dimensions and definitions, and its complexity may be the cause of confusion, but the benefits of its inclusion into the students' curriculum are irrefutable. An encompassing definition of "bilingualism" is an individual who has native proficiency in one language and proficiency in a second language.

Research has shown that bilingual education programs will increase academic achievement, self-esteem and social adjustment for students (Garcia 42). Schools that choose to incorporate bilingual programs as part of the curricular objectives will not only benefit students whose primary language is not English but also bring about changes in educational outcomes that will benefit the entire American society. Society at large will reap the benefits of a multicultural and multilingual environment.

Throughout the past three years, I have been actively engaged in an ECG program, which bridges Cabrini College with the Norristown Area School District. I have had the opportunity to work with the Middle School and the High School Latino students. Through this program I can personally attest to what other researchers have also shown --bilingual education an interaction that respects and includes other cultures is extremely advantageous.

There is ample research that indicates bilingual education gives the students social, linguistic and cognitive advantages over their peers. Bilingual children have outperformed their monolingual peers in numerous studies. Bilingualism is a form of brain insurance that keeps your brain sharp throughout the years. Bilingual students comprehend the complexities of language and are more adept to correct errors in language meaning and grammar. They also tend to demonstrate a highly developed ability to vary word usage based on the needs of the listener. Bilingualism also refines students' cognitive abilities, which include a better comprehensive knowledge of visual problem solving skills and analytical tests. Bilingual students gain social advantages over their monolingual peers as well. They are more comfortable in a multicultural environment and tend to be more open minded, tolerant and culturally competent towards people, languages and cultures.

Bilingual students show definite advantages over monolingual students in four cognitive and learning areas, which include: cognitive flexibility, metalinguistic awareness, communicative sensitivity and field independence (Garcia 49). Cognitive flexibility indicates that the bilinguals are more creative and flexible in their thought processes. Cognitive flexibility is divided into two subcategories, which include divergent and convergent thinking. Divergent thinking is measured by providing an individual with a starting point and asking them to generate a series of permissible solutions. Convergent thinking is measured by tests that provide a number of pieces of information that an individual must synthesize to arrive at the correct answer. Researchers report that bilingual individuals are superior to their monolingual counterparts in both the divergent and convergent thinking. Bilingual individuals also demonstrate a greater awareness of language and how it works which is referred as metalinguistic awareness.

Metalinguistic awareness is the ability to analyze language and particularly language forms, in terms of how they work and how they are integrated into the wider language system. Phonological awareness is one level of metalinguistic awareness, which is the understanding of sound units, word awareness, and syntactic awareness. Bilingual students have a greater metalinguistic awareness because they are working with more than one language simultaneously which requires a greater awareness of the similarities and differences of each language.

Processing the sounds and words of different languages is difficult but studies indicate that the hard work pays off due to the increase in the gray matter of the brain. The gray matter is the part of the brain that controls language and communication, and a higher density has been correlated with increased intellect. Many individuals argue that bilingual education creates confusion in younger brains, but science indicates that the discipline required to switch between languages improves the child's ability to focus on an individual task. The bilingual students also have better self-regulation, which helps them exceed their monolingual peers in school (citation).

Research also indicates that the left hemisphere is dedicated to the acquisition of language and communication. Although bilingual brains have shown through experimentation that the two languages have two subsets of neural connections, one for each language. The languages can be activated or inhibited independently of each other, but at the same time both languages are easily retrievable.

This idea allows bilingual individuals to speak in one language and understand what is being said in another. This proves that the phonological production and perception systems of language are independent and provides an outstanding advantage over individuals who are fluent in only one language. Bain and Yu measured the cognitive ability of infants over the course of 48 months, one set with monolingual acquisition and the other with bilingual acquisition. These infants were subjected with the approval of their parents to a series of cognitive tests.

During the first and second sets of testing there was no significant difference between the monolingual and the bilingual infants. However, during the third set of testing when the children were between 46 and 48 months old the results showed that the bilingual children outperformed their monolingual peers (Romaine 106).

Communicative sensitivity explains how bilingual individuals are more sensitive to nuances in communication. Bilingual individuals tend to be more communicatively aware because they are used to thinking about switching between two different languages. They also have to listen and pick up clues about when to switch between languages when talking to different people. A bilingual person has an increased sensitivity to the social nature and communicative functions of a language.

There have been numerous studies that exemplify the benefits of bilingual education yet it is still viewed negatively by many people in the U.S. The bilingual brain has been studied through various experiments resulting in the positives and negatives of bilingualism on cognitive development. One study indicates the students having difficulty with code switching, mixing the two languages together. Many individuals view this study and fear that the native language of children will be confused and cease to exist. On the contrary to the results of this study, the intertwining of two languages proves the ability of the brain to retain the languages making the student well versed and culturally aware of others (Romaine 111).

The last cognitive advantage of bilingual students states that they have more field independence, which refers to the ability to orient themselves and detect hidden patterns and figures more easily. Field independence increases as children grow and mature. Bilingual students tend to be more field independent at a younger age compared to their monolingual peers. Evidence collected over time has determined that there are many other advantages for bilingual students besides an increase in cognitive development; there are clearly social advantages.

Bilingual students are able to communicate with a vast majority of Spanish speaking and English speaking individuals. The bilinguals can associate with more than one community, which gives them a social advantage compared to their peers. They are also more culturally competent and sensitive towards people, languages and cultures.

Due to their ability to speak two languages they are more comfortable in a multicultural environment and tend to be more open minded and tolerant. Bilinguals gain confidence in a multicultural environment because they not only adopt a second language but a second culture as well. Their knowledge and understanding of two languages and cultures allows them to identify with both cultures.

Schools that choose to incorporate bilingual programs as part of the curricular objectives will not only benefit students whose primary language is not English but also bring about changes in educational and social outcomes that will benefit the entire American society. Society at large will reap the benefits of a multicultural and multilingual environment. Students who are immersed in bilingual education are experiencing two cultures merge, and define who they are as an individual. These individuals can identify

with both Spanish and English-speaking individuals, which gives them a greater sense of belonging. Learning two languages and cultures reduces the risk of racism and prejudice in the classrooms. When an individual can identify themselves with two different cultures it increases social acceptance. With an increase in social acceptance there will be a decrease in discrimination, which is a huge problem in society today.

Throughout the past three years I have been actively engaged in an ECG program, which bridges Cabrini College with the Norristown Area School District. I have had the opportunity to work with the Middle School and the High School Latino students. Through this program I can personally attest to what other researchers have also shown -- bilingual education and interaction is extremely advantageous.

In this program I have increased my knowledge of the Hispanic culture and language. Two professors from the Romance Languages and Literatures Department started this program in 2009 and have established strategic relationships with NASD's higher-level administration and staff, both at the High School and Eisenhower Middle School. Through strategic relationships it has become possible for the ECG students to mentor ELL students to help improve graduation and retention rates. Our goal is to sustain the program and let it grow and flourish in the years to come. As mentors for the Latino students we are not only trying to help them succeed in education but give them a voice in the school district.

Many of the Latino students are placed in English-only classrooms, which reinforce the language barrier between themselves, the teacher and many of their peers. Students participating in the Cabrini Bridges Communities Mentoring Program are advocates for the Latino students, their families and the community. We strive to improve graduation and retention rates but we are also advocating for greater social justice in the process.

We speak for the Latino community in Norristown, for those who are silenced because of the language barrier. It is both unjust and unacceptable to silence someone because they do not speak the same language. I advocate for bilingual education, for the same educational rights for the Latino students, and for the increase and support of culturally competent and sensitive teachers.

Works Cited

García, Eugene, et al. 1946. Bilingualism and Cognition: Informing Research, Pedagogy and Policy. York, PA. Library of Congress.

Hernandez, Arturo, 2013. The Bilingual Brain. New York, NY. Oxford University Press.

Romaine, Suzanne, 1989. Bilingualism. New York, NY. Oxford University Press.

Horacio Quiroga y la cuentística del amor, la locura y la muerte

Author: Thomas Hale

Faculty Mentor: Raquel A. Green, PhD

Abstract: This essay explores the themes, structure and narrative techniques of two iconic short stories of the Argentinian short story writer, Horacio Quiroga. The essay demonstrates how Quiroga´s personal obsession with love, insanity, and death permeates his stories and dictates the use of narrative techniques that propels the reader into a world of despair and death.

La vida del cuentista uruguayo Horacio Quiroga estuvo llena de tragedia y muerte, por eso fue natural que él escribiera de estos temas. Durante la escritura de su antología *Cuentos de amor, de locura y de muerte*, Quiroga vivía con su esposa en un lugar remoto de la provincia de Misiones. Se especula que en ese entonces, el escritor estaba obsesionado en educar sus hijos para sobrevivir la vida cruda de la selva. Aparentemente deprimida y descontenta con la conducta de Quiroga, su esposa se termina suicidando. La presencia de la tragedia durante su vida corresponde a la realidad de la muerte como tema en muchas de sus obras, en particular los dos cuentos que voy a considerar en este ensayo.

"El almohadón de plumas" y "A la deriva." Según el crítico literario Noé Jitrik, la obsesión de Quiroga de escribir sobre estos temas se debe a que "la única manera que se le ocurre para expulsarla es escribirse muerto, única arma con que cuenta para conjurar y deshacer la imagen que lo atemoriza y cohíbe" (Alonso 110).

Otro tema y elemento que aparece en sus obras es la naturaleza; de hecho, Quiroga eligió a Misiones, una región de selvas salvajes en el norte de Argentina como escenario de sus cuentos más famosos. Sus intereses variados hacen comprender las motivaciones detrás de los temas que selecciona para escribir los cuentos que, en concordancia con sus propias reflexiones críticas sobre el ejercicio narrativo, tienen una intensa acción combinada con conclusiones psicológicas más profundas.

De acuerdo con su propio "Decálogo del perfecto cuentista," Quiroga subraya la importancia de la exposición, la conclusión y una acción escrita con la precisión de un inventor, cuyos temas oscuros hablan de una persona familiarizada con la muerte (Decálogo n.p.).

En su cuento "El almohadón de plumas," Quiroga usa el tiempo narrativo en conjunto con los tiempos verbales para insertar ráfagas espásticas a la trama con el fin de crear tensión en lector al reflejar los cambios emocionales asociados con el lento descender hacia la muerte de Alicia, la joven que agoniza en plena luna de miel. El narrador abre el cuento con las palabras, "su luna de miel fue un largo escalofrío" que duró "tres meses," y luego añade que ellos "se habían casado en abril."
Desde la perspectiva de un hispanoamericano, el mes de abril indica el comienzo del otoño, una estación en que las temperaturas bajan y la luz disminuye. Si según el narrador ya habían pasado tres meses, entonces las acciones principales del cuento ocurrían en la mitad del invierno. El simbolismo del "escalofrío" indica los cambios de estación pero también prefigura una enfermedad.

De hecho, más tarde en el cuento, Quiroga dice que Alicia "tuvo un ligero ataque de influenza que se arrastró insidiosamente días y días". Otras señales temporales sirven para exagerar la intensidad de las acciones de Jordán. En el primer párrafo del cuento, el narrador describe momentos en que Alicia mira fijamente a Jordán "mudo desde hacía una hora;" también la voz narrativa confirma que Jordán tiene un temperamento reservado cuando dice él "la amaba profundamente, sin darlo a conocer." La descripción breve de los tres meses de su luna de miel se contrasta con la descripción larga del decaimiento de Alicia que tuvo lugar en el lapso de "cinco días" y "cinco noches."

En los párrafos donde se incluye la descripción de cómo progresa la enfermedad, el escritor usa principalmente el tiempo verbal del pretérito para exagerar la brusquedad de la enfermedad que Alicia había contraído. Pero después de la visita del médico que le advierte a Jordán "si mañana se despierta como hoy, llámeme enseguida", Quiroga introduce la urgencia de que algo trágico va a pasar.

El lector debe adaptarse a los tiempos del narrador que inmediatamente dice, "Al otro día Alicia seguía peor" y describe que la joven "se iba visiblemente a la muerte." Después de este momento de intensidad narrativa, Quiroga demora la velocidad del cuento con el uso de verbos en el imperfecto y mueve la descripción de Jordán y Alicia diciendo que ella "dormitaba" mientras él "vivía casi en la sala" "paseábase sin cesar" y "a ratos entraba en el dormitorio…mirando a su mujer cada vez que caminaba en su dirección". La acción sugerida por el lenguaje verbal se acelera de nuevo cuando ella "comenzó a tener alucinaciones."

Aunque Jordán estaba paseando por los pasillos, él "corrió al dormitorio" para ver cuando "Alicia dio un alarido de horror." Quiroga usa los verbos en pretérito para destacar la naturaleza espasmódica de la condición de Alicia. Él escribe que "Alicia lo miró con extravió, miró la alfombra, volvió a mirarlo, y después de largo rato de estupefacta confrontación, se serenó."

Como se mencionó anteriormente, la presencia de la naturaleza y su poder es un rasgo clave en muchas obras de Quiroga. En "El almohadón de plumas," Quiroga usa las estaciones para marcar el tiempo narrativo y añadir una dimensión simbólica que se refleja en la acción del cuento. Además de las estaciones, Quiroga permite que el lector crea que Alicia sufría y moriría de una influenza severa.

Quiroga no revela hasta el párrafo final la causa verdadera y causante de la muerte que se esconde dentro de la almohada de Alicia. Al final del cuento, la voz narrativa se dirige al lector en el presente describiendo que "Estos parásitos de las aves, diminutos en el medio habitual, llegan a adquirir, en ciertas condiciones, proporciones enormes. La sangre humana parece serles particularmente favorable, y no es raro hallarlos en los almohadones de pluma" (Quiroga).

La causa de su muerte no es detectada hasta después que Alicia muere. Se puede vincular este desenlace a la experiencia misma del autor, que experimentó mucha tragedia durante su vida, y también despierta horror y sospecha –precisamente lo que Quiroga desea ofrecer al lector.

La naturaleza es también la fuerza central del cuento "A la deriva," en que los elementos naturales como el río y la serpiente venenosa "yaracacusú" tienen tanto poder como el anónimo personaje principal. El cuento empieza cuando "el hombre pisó algo blancuzco" que es identificado como una "yaracacusú." Quiroga describe que la serpiente deja "dos gotitas de sangre." En este relato también, Quiroga introduce otros elementos naturales activos, como el río Paraná, que representa la fuerza natural que físicamente guía la acción.

De esta manera seguimos las acciones a través de marcadores narrativos como "el Paraná corre," "el sol había caído," "el veneno comenzaba a irse," y "el bienestar avanzaba." Quiroga hace que el protagonista vaya perdiendo lentamente el control de las funciones de su cuerpo, y esta técnica le sirve para vivificar la descripción, insertar tensión en la lectura y llevar el relato hacia adelante. Sin embargo, Quiroga deja saber a los lectores lo que siente el protagonista.

Como en otros cuentos de Quiroga, la trama de "A la deriva" sirve para exagerar la decadencia de los personajes centrales que terminan en la muerte. Debido a que el lector puede predecir el desenlace del relato, Quiroga voltea el reloj de arena y lo entretiene mientras el personaje se va muriendo. Después del ataque de la yaracacusú, a pesar de los esfuerzos del hombre por aislar el veneno, la primera sensación de debilitamiento es la del sabor, porque cuando la mujer en el rancho le trae una fuerte bebida alcohólica, él protesta "¡No, me diste agua! ¡Quiero caña, te digo!" y el narrador confirma muy pronto que "el hombre tragó uno tras otro dos vasos [de damajuana], pero no sintió nada en la garganta."

El hombre continúa a pesar de no poder saciar su sed porque él "no quería morir." Con la intención inicial de viajar "cinco horas a Tacurú-Pucú" el hombre "[palea] hasta el centro del Paraná," pero "allí sus manos dormidas dejaron caer la pala en la canoa" cuando su bajo vientre se "desbordó hinchado, con grandes manchas lívidas y terriblemente doloroso." El hombre cambió su plan y "se decidió a pedir ayuda a su compadre Alves."

En su favor, "la corriente del río se precipitaba ahora hacia la costa brasileña" donde le gritó a Alves pero, "en el silencio de la selva no se oyó un sólo rumor." El sol "había caído" indicando al lector el fin del día y causando al hombre tener un "violento escalofrío" y esta pista ya indica que mientras "la pierna le dolía apenas, la sed disminuía, y su pecho, libre ya, se abría en lenta inspiración", él no se iba a recuperar. Quiroga con gran destreza usa las funciones corporales para hacer creer al lector que por lo menos mentalmente todo sigue funcionado, ya que el personaje se lleva a cuestionar cuando conoció a su antiguo patrón. Pero cuando abruptamente, el narrador explica ese diálogo interno, logra darse la respuesta mientras el personaje "cesó de respirar."

En el análisis de estos dos cuentos ejemplares, "El almohadón de plumas" y "A la deriva," Quiroga hace ejercicio de las mejores reglas expuestas en su "Decálogo al perfecto cuentista." Aunque en los cuentos falta acción dramática constante, Quiroga prolonga con maestría el sufrimiento de los protagonistas y ofrece otros elementos para guiar la trama y entretener al lector. Por ejemplo, en "El almohadón de plumas, " Quiroga oscila entre los tiempos verbales imperfectos y pretéritos para sugerir el deterioro de la salud de Alicia.

En "A la deriva, " la acción principal ocurre en el comienzo y el papel de los elementos naturales, como el veneno de la serpiente, el sol poniente, y el río guían el relato a su meta, mientras la salud del protagonista se va desintegrando.

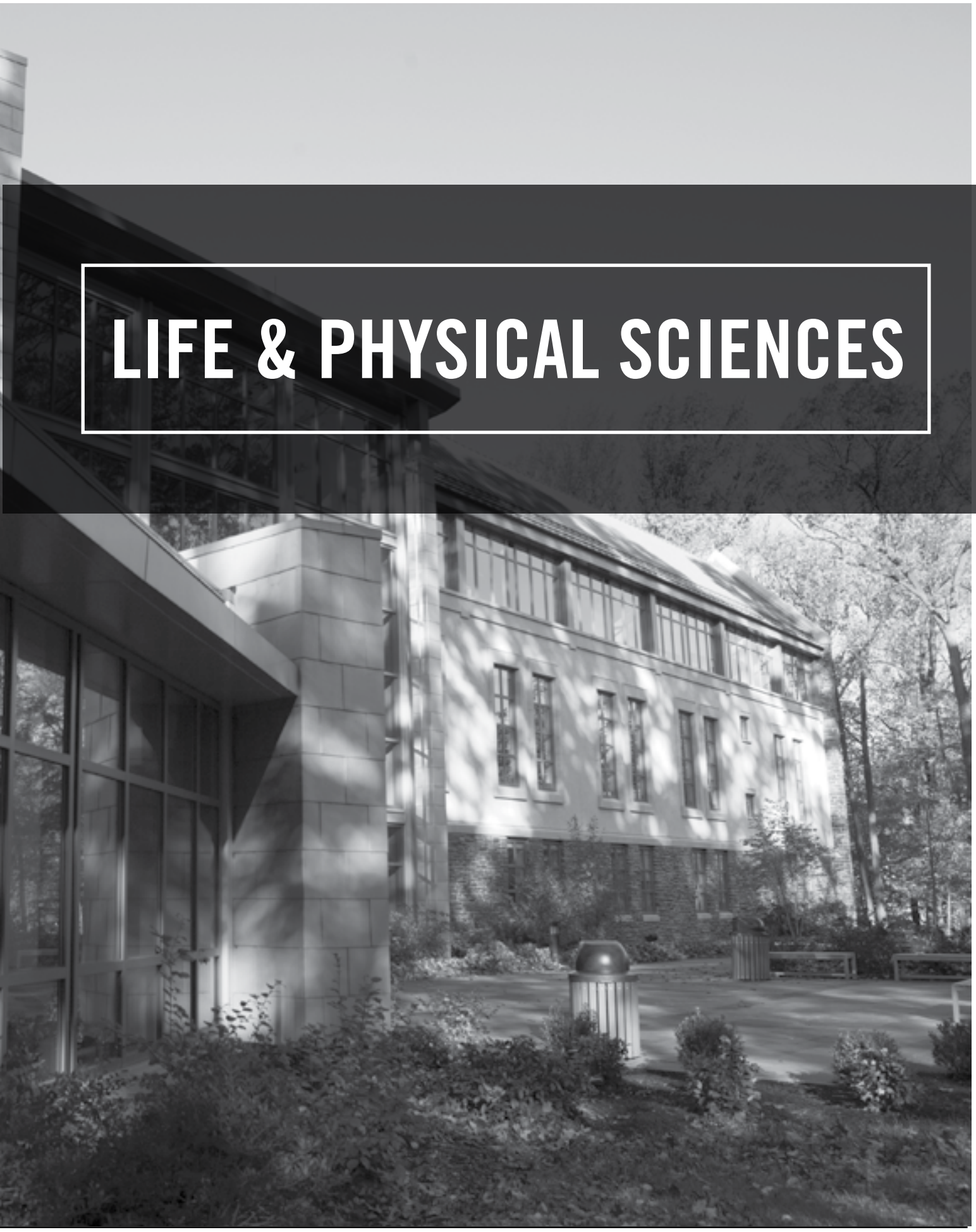
Ambos cuentos terminan en forma inesperada, y el lector siente que ha sido llevado de vuelta a la realidad después de un viaje surrealista. De esta manera, Quiroga ejemplifica el dominio de la narración que describe en su famoso "Decálogo del perfecto cuentista," él logra que el lector también se obsesione en la conclusión con la muerte.

Works Cited

Alonso, Carlos J. *The Burden of Modernity the Rhetoric of Cultural Discourse in Spanish America*. New York: Oxford UP, 1998. Print.

"Decálogo Del Perfecto Cuentista - Horacio Quiroga - Ciudad Seva." *Decálogo Del Perfecto Cuentista - Horacio Quiroga - Ciudad Seva*. Web. 2 Dec. 2014. <http://www.ciudadseva.com/textos/teoria/opin/decalogo_del_perfecto_cuentista.htm>.

French, Jennifer. *Nature, Neo-colonialism, and the Spanish American Regional Writers*. 1.st ed. Hanover, N.H.: Dartmouth College, 2005. Print.



LIFE & PHYSICAL SCIENCES

Proteogenomic refinement of mycobacteriophage Catdawg reveals non-canonical novel genes

Authors: Courtney Clyburn, Dylan Chudoff, Jennifer Farina, and Michelle Zajko
Faculty Advisor: David Dunbar, PhD, Professor of Biology Professor

Keywords: proteomics, mycobacteriophage, SEA-PHAGES, novel phage genes

Abstract: We have demonstrated conducting mass spectrometry analysis on mycobacteriophage Catdawg lysate using the bioinformatics program VESPA the expression of 66 open reading frames (ORFs) with two or more unique peptides. Of these, there are two non-canonical novel ORFs that overlap and are expressed in the opposite orientation of ORFs with known functions. An additional novel ORF is located "out of frame" of a previously annotated ORF. Intriguingly, there are single peptide matches to 26 additional genomic sites that contain at least one possible start codon suggesting that previously unidentified non-canonical ORFs are abundant within the Catdawg genome. Using validated orphan peptide data we were also able to demonstrate the expression of an additional 15 previously annotated ORFs. During the course of our orphan peptide analysis we were also able to confidently reassign the start sites of two genes (gp41 and gp101).

Introduction

Previous studies have shown that there are 1 x 10^{31} bacteriophages globally with an estimated (Wommack & Colwell, 2000). Furthermore, the entire bacteriophage population is thought to turn over every few days (Suttle, 2007). Based on the sheer number of bacteriophages, they serve as a largely untapped reservoir of genes, many with unknown functions (Hendrix, 2003). The largest collection of sequenced and genomically characterized bacteriophage genomes are those of the mycobacteriophages (Pope et al., 2015).

Of these all but a few of them can infect the common host *Mycobacterium smegmatis* mc^2155. Mycobacteriophages are classified in different clusters based on similarity at the genomic level. Based on the recent data on www.phagesdb.org website there are currently 23 mycobacteriophage clusters (A-W) and of these clusters, several of them can be further subdivided into two or more subclusters based on genomic comparisons as of this writing. Bioinformatic analysis can be used to assign the function for many of the structural genes as they have similarity to structural genes of other bacteriophages. However, there are large numbers of non-structural genes located within mycobacteriophage genomes whose functions are hard to predict bioinformatically.

Many of these genes also have no apparent homologs in other bacteriophage or bacterial genomes. Using a program called phamerator, many of the structural and non-structural genes found in mycobacteriophage genomes code for similar homologous proteins using a program that can be grouped into phamilies based on amino acid sequence homology (Cresawn et al., 2011).

Presumably, proteins grouped in the same phamily have the same or similar function(s). Protein encoded mycobacteriophage genes are predicted using Glimmer and Genemark gene annotation software tools embedded in a program called DNA Master (http://cobamide2.bio.pitt.edu/). Direct experimental evidence is lacking for the majority of predicted genes in most of the hundreds of mycobacteriophage genomes that have been published in GenBank.

Because of the enormity of the number of mycobacteriophage genomes and their predicted gene products it is most likely that bioinformatics approaches alone do not identify all of the ORFs contained within mycobacteriophage genomes. Furthermore, annotation software can make incorrect start site predictions of correctly predicted ORFs. Recent proteomic analysis of mycobacteriophage Patience of purified virions or phage infected cells revealed the presence of 79/109 predicted gene products (Pope et al., 2014).

Of these genes, 7 of them were misannotated. Three gene products were newly identified using proteomics analysis and were previously missing from the annotated genome. Intriguingly, there were peptides that matched several "out of frame" regions of annotated ORFs. Furthermore, these "out of frame" regions have predicted strong ribosome sites associated with potential start codons.

Proteomic analysis was also conducted on two O cluster mycobacteriophages, Corndog and Catdawg. There are currently five O cluster phages and all five of them have a characteristic prolate head. Proteomics conducted on purified mycobacteriophage Corndog virions or Corndog infected cells confirmed the identity of 60 previously annotated ORFs, confirmed the start sites of 25 ORFs, reassigned the start site of number three genes and identified two additional previously unannotated ORFs.

In the same study, proteomic analysis was conducted on an unpurified lysate mycobacteriophage Catdawg and revealed the expression of 63 Catdawg genes. Of these, the start sites of 26 genes were confirmed and the start site of one additional gene (gp122) was reassigned.

Results & Discussion

Because proteomic analysis identified two additional previously unidentified ORFs located within the Corndog mycobacteriophage genome, a related O cluster phage (Cresawn et al., 2015) we deemed it important to determine if additional proteomics analysis would identify any previously unidentified ORFs in the Catdawg genome.

Our previous proteomic analysis using the program Scaffold was used to match peptides to previously annotated ORFs as published in GenBank. To identify any potential novel ORFs and attempt to confirm or reassign the start sites of additional expressed gene products, we identified peptides that matched a six frame translation of the Catdawg genome. Matching peptides were then used in a program called VESPA to identify regions of the Catdawg genome contained matching peptides.

VESPA is a tool that was originally developed to be used to identify high throughput peptide and transcriptomic (RNA-Seq) data matches to regions of microbial genomes (Peterson et al., 2012). We reasoned that VESPA would also be a valuable tool to use for the identification of novel ORFs and/or to assist in the mapping of the start sites of identified ORFs contained within bacteriophage genomes as well.

Figure 1 represents a VESPA analysis in the Reading Frame Visualization view of the Catdawg genome. Displayed are the annotated ORFs published in Genbank as blue boxes, peptide matches to annotated ORFs as red bars and peptide matches to regions outside of annotated ORFs or extending beyond annotated ORFs in yellow. Potential coding regions that match peptides that were not annotated as genes are shown as light green boxes.

Table 1 lists annotated genes and whether gene products encoded for each gene were identified via VESPA analysis. Identified functions for annotated ORFs were also noted using bioinformatics tools ((Cresawn et al., 2015) and this work. Using HHpred (Söding, Biegert, & Lupas, 2005) and NCBI Blast algorithms we can only confidently identify the functions and/or functional domains for 25/128 annotated ORFs.

Altogether, we identified the expression of 66 annotated ORFs with two or more unique peptide matches. Sixty three of them were identified by proteomics analysis and published in previous work (Cresawn et al., 2015). As can be seen in Figure 1, several ORFs have peptide sequence coverage over greater than 90% of the sequence. The majority of high sequence coverage ORFs encode identified structural gene products with known functions (Figure 1 & Table 1).

These include, among others, the major capsid subunit (gp39), the major tail subunit (gp47) and the tape measure gene (gp54). In this work we identified the expression of three additional ORFs (gp108, gp101 & gp128) using the criteria of two unique peptides per ORF. We also detected high confidence single unique peptide matches for fifteen additional previously annotated ORFs (Figure 1). Thus, based on our current analysis we confidently predict the presence of 81/128 previously annotated ORFs located within the Catdawg genome as published in GenBank.

Peptides that either matched areas outside of an annotated ORF or spanned an annotated ORF were manually validated to ensure confidence that these peptides were not false positives. Peptides that did not meet the validation criteria were not used in this analysis.

Forty seven out of seventy five peptides met a high confidence manually validated peptide standard. Intriguingly, we identified high confidence validated peptide matches to 30 regions of the Catdawg genome not matching any of the annotated ORFs (shown in Figure 1 as light green boxed areas).

Some of these candidate novel ORFs appear to be “out of frame” embedded within regions of previously annotated ORFs. Other candidate novels ORFs overlap previously annotated ORFs but are oriented in the opposite direction (Figure 1).

Three of these candidate novel ORFs contain two or more validated peptide matches. In all cases but one instance, there is at least one potential start codon located upstream of the matching peptide. A peptide that matches potential novel ORF gp4329 does not have any identifiable upstream start codon. Table 2 depicts the peptide sequences along with coordinates for all possible start codons located within peptide genomic matching regions for all potential novel ORFs.

Shown also in Table 2 are the corresponding Shine Dalgarno (SD) sequences associated with possible start codons and associated final SD scores based on the Kibler SD scoring matrix (Hampson & Kibler, n.d.; Kibler & Hampson, n.d.).

Final SD scores are based on the possible SD sequence and spacing between the putative SD and start codon. The higher the final SD score, meaning the negative the number, the higher the confidence is given for any particular potential SD sequence. The parameters set in DNA Master for the analysis of SD regions associated with possible start codons is Kibler 6 and Karlin medium.

BlastP or HHpred analysis using the sequence of the longest possible amino acid sequence for each candidate ORF is not informative as to the possible function(s) for possible gene products. Nor does bioinformatic analysis detect homology to gene products from any other organism. This is not to be totally unexpected since the majority of annotated ORFs, many of which are shown to be expressed, cannot be assigned a definitive function bioinformatically.

We are confident that at least three of these candidate novel ORFs code for protein products since they have two or more unique peptide matches to the designated region. Two of these novel ORFs are oriented in the leftwards direction and overlap genes that are oriented in the rightwards direction. For instance, novel ORF gp4461 appears to completely span gp66, the lysA protease gene (Figure 1). Five unique validated peptides match gp4461 (Table 2).

Novel ORF gp4561, for which there are two unique validated peptides (Table 2), completely overlaps the last third of gp54, the tape measure protein (TMP) (Figure 1). An additional novel ORF gp3338 is oriented in the rightwards direction and contains two unique peptide matches contained within the genome that consist of a clustered set of leftward oriented genes.

VESPA analysis also merits start site reassignment for two previously annotated ORFs (gp41 & gp101). For gp41, several orphan peptides span the annotated start site upstream five amino acids from the misannotated start site next to a methionine residue, the correct start site for this gene (Figure 2). VESPA analysis also reveals that gp101 has been misannotated since several orphan peptides match areas upstream of the annotated start site. The only possible upstream start site is an ATG codon that is located 72 base pairs upstream of the misannotated start site.

For another ORF (gp124), orphan peptide data suggests that the correct start site is located 327 nucleotides upstream at a GTG start codon at nucleotide position 69932. Figure 1 indicates that this start site completely overlaps two additional ORFs in the minus two reading frame (gp125 & gp126). However, we are cautious in reassigning the start site for gp124 since we have not identified any peptide matches to the annotated region of gp124.

Validated peptide analysis suggests that candidate novel "out of frame" ORFs and candidate novel ORFs that span known ORFs oriented in the opposite direction seem to be abundant within the Catdawg genome. Whether or not these candidate novel ORFs code for functional gene products is unknown.

Work by (Liu, Jiang, Gu, & Roberts, 2013) using ribosomal profiling with phage lambda, identified novel expressed "out of frame" ORFs imbedded within previously identified ORFs and novel ORFs that overlap ORFs but are expressed in opposite directions.

This suggests that unexpected novel ORFs might be more pervasive in phage genomes then currently thought. However, we cannot rule out the possibility that non-canonical novel gene products have no functional or regulatory role but instead represent spurious background gene expression. It is also possible that these candidate novel ORFs do represent gene products with important but unknown functions. We note that most of these presumed novel ORFs would code for small protein products.

As indicated by (Hobbs, Fontaine, Yin, & Storz, 2011) many small proteins encoded in phage genomes have been understudied and are revealing important roles in lysogeny, phage growth and as bacterial toxins. Clearly, more work needs to be done to provide additional convincing evidence that these presumed novel ORFs are expressed or whether the majority of them represent background expression "noise" and nothing more.

Since we identified Catdawg gene products employing proteomics using a phage lysate we are unsure when the possible identified novel gene products are expressed and function during a phage infection cycle. Future work is aimed at conducting

additional proteomics studies during different time points post phage infection to provide additional convincing evidence that the putative novel ORFs are expressed and at what stage of the phage infection cycle gene products are detected.

Additional work is also aimed at determining RNA expression levels of gene products using RNA-Seq and Ribosomal profiling transcriptomic methods using RNA from different time points post phage infection.

Expression level data over a time course of infection should also help reveal whether identified novel ORFs are expressed at high, low or background levels, relative to other known phage structural and regulatory proteins offering additional clues as to their functional importance. However, even proteins expressed at low levels might have important roles to play.

Together, additional proteomics and transcriptomics analysis with phage Catdawg and related phages should help to provide more evidence whether phage genomes have more complex gene expression patterns than previously realized.

Materials & Methods

Proteomic Analyses

Mass spectrometry on a Catdawg lysate was described in (Cresawn et al., 2015). There were a total of 4055 non-redundant matched unique peptides that matched a six frame translation of the Catdawg genome with a probability of 80% or greater. The unique peptides that matched the Catdawg genome were used in a program called VESPA (Peterson et al., 2012) to match peptides to regions of the Catdawg genome. VESPA allows one to identify peptides that match annotated ORFs as well as regions of the genome outside of annotated ORFs. Peptides that either matched areas outside of an annotated ORF or single peptides that matched annotated ORFs were manually validated to ensure confidence that these peptides were not false positives. Peptides that did not meet validation criteria were not used in this analysis.

Bioinformatic Analyses

Candidate Catdawg novel ORFs were analyzed using DNA Master (http://cobamide2.bio.pitt.edu/). Imbedded within DNA Master are the gene searching tools , GLIMMER (Delcher, Harmon, Kasif, White, & Salzberg, 1999) GeneMark (Borodovsky, 2011) and BLAST. HHpred (Söding et al., 2005) was also used for protein function analysis.

References

Cresawn, S. G., Bogel, M., Day, N., Jacobs-Sera, D., Hendrix, R. W., & Hatfull, G. F. (2011). Phamerator: a bioinformatic tool for comparative bacteriophage genomics. *BMC Bioinformatics*, *12*(1), 395. http://doi.org/10.1186/1471-2105-12-395

Cresawn, S. G., Pope, W. H., Jacobs-Sera, D., Bowman, C. a, Russell, D. a, Dedrick, R. M., … Hatfull, G. F. (2015). Comparative genomics of cluster o mycobacteriophages. *PloS One*, *10*(3), e0118725. http://doi.org/10.1371/journal.pone.0118725

Delcher, a L., Harmon, D., Kasif, S., White, O., & Salzberg, S. L. (1999). Improved microbial gene identification with GLIMMER. *Nucleic Acids Research*, *27*(23), 4636–41. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=148753&tool=pmcentrez&rendertype=abstract

Hampson, S., & Kibler, D. (n.d.). Evaluating Representations for the Shine-Dalgarno Site in Escherichia coli. *Citeseer*. Retrieved from http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.15.4664&rep=rep1&type=pdf

Hendrix, R. W. (2003). Hendrix_bacteriophage genomes.pdf. *Current Opinion in Microbiology*, *6*, 506–511.

Hobbs, E. C., Fontaine, F., Yin, X., & Storz, G. (2011). An expanding universe of small proteins. *Current Opinion in Microbiology*, *14*(2), 167–173. http://doi.org/10.1016/j.mib.2011.01.007

Kibler, D., & Hampson, S. (n.d.). Characterizing the Shine-Dalgarno Motif: Probability Matrices and Weight Matrices. *Science*.

Liu, X., Jiang, H., Gu, Z., & Roberts, J. W. (2013). High-resolution view of bacteriophage lambda gene expression by ribosome profiling. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(29), 11928–33. http://doi.org/10.1073/pnas.1309739110

Peterson, E. S., McCue, L. A., Schrimpe-Rutledge, A. C., Jensen, J. L., Walker, H., Kobold, M. a, … Webb-Robertson, B.-J. M. (2012). VESPA: software to facilitate genomic annotation of prokaryotic organisms through integration of proteomic and transcriptomic data. *BMC Genomics*, *13*(1), 131. http://doi.org/10.1186/1471-2164-13-131

Pope, W. H., Bowman, C. a, Russell, D. a, Jacobs-Sera, D., Asai, D. J., Cresawn, S. G., … Hatfull, G. F. (2015). Whole genome comparison of a large collection of mycobacteriophages reveals a continuum of phage genetic diversity. *eLife*, *4*. http://doi.org/10.7554/eLife.06416

Pope, W. H., Jacobs-sera, D., Russell, D. A., Rubin, D. H. F., Kajee, A., Msibi, Z. N. P., … Hatfull, F. (2014). Genomics and Proteomics of Mycobacteriophage Patience , an Accidental Tourist in the Mycobacterium Neighborhood, *5*(6), 1–11. http://doi.org/10.1128/mBio.02145-14. Editor Söding, J., Biegert, A., & Lupas, A. N. (2005). The HHpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Research*, *33*(Web Server issue), W244–8. http://doi.org/10.1093/nar/gki408

Suttle, C. a. (2007). Marine viruses--major players in the global ecosystem. *Nature Reviews. Microbiology*, *5*(10), 801–812. http://doi.org/10.1038/nrmicro1750

Wommack, K. E., & Colwell, R. R. (2000). Virioplankton: viruses in aquatic ecosystems. *Microbiology and Molecular Biology Reviews : MMBR*, *64*(1), 69–114.
Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=98987&tool=pmcentrez&rendertype=abstract

Acknowledgements: We thank the Cabrini College Science Undergraduate Research Funds for generously providing funds that supported this work.

Figure 2. VESPA sequence view of orphan peptides indicating a misannotated start site for gp39.

Sequence view of the region highlighted in Figure 2. The orphan peptides appear in yellow and can be observed spanning the presumed start site of gp39.

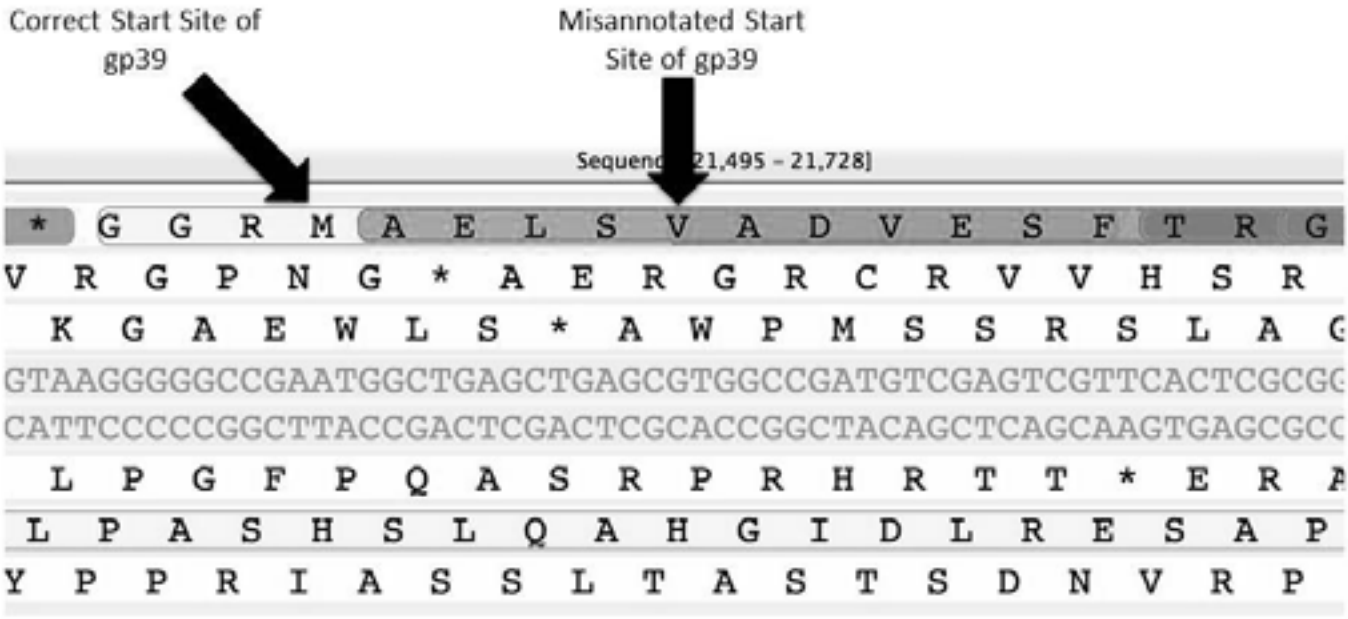


Table 1. List of Catdawg annotated genes. ORFs with gene products detected in both a prior study and this study are indicated with an asterisk, ORFs with gene products detected in this study are indicated with a two asterisk designation. ORFs with single peptide matches are designated with a (1) after the gene name.

Gene Product	Gene Function
gp1*	-
gp2(1)**	-
gp3	-
gp4*	-
gp5	DNA Methylase
gp6(1)**	DNA Methylase
gp7	-
gp8	-
gp9*	Endonuclease
gp10(1)**	-
gp11*	-
gp12*	-
gp13	-
gp14*	-
gp15*	-
gp16*	-
gp17	-
gp18	-
gp19*	Primase/Polymerase
gp20	-
gp21*	-
gp22	-
gp23	-
gp24*	-
gp25	-
gp26	-
gp27(1)**	HNH Endonuclease
gp28	-
gp29	-
gp30(1)**	Terminase Large Subunit
gp31	-
gp32*	Portal
gp33*	O-Methyl-Transferase
gp34*	-
gp35*	Glycosyltransferase
gp36*	Glycosyltransferase
gp37*	Prohead Protease
gp38	-
gp39*	Major Capsid
gp40*	-
gp41*	-

gp42	-
gp43*	-
gp44*	-
gp45*	-
gp46*	-
gp47*	Major Tail
gp48	-
gp49(1)**	-
gp50*	-
gp51*	-
gp52*	Tail Assembly Chaperone
gp53	Tail Assembly Chaperone
gp54*	Tapemeasure Protein
gp55*	Minor Tail Protein
gp56*	Hydrolase Domain
gp57*	-
gp58*	-
gp59*	-
gp60*	-
gp61*	-
gp62*	-
gp63*	Peptidoglycan Recognizing Protein Domain
gp64*	Hydrolase Domain
gp65	-
gp66*	LysA
gp67*	LysB
gp68	Holin
gp69*	-
gp70	-
gp71(1)**	-
gp72*	-
gp73*	DNA Binding Domain
gp74*	-
gp75(1)**	-
gp76(1)**	-
gp77(1)**	-
gp78*	-
gp79*	-
gp80*	-
gp81*	DNA Polymerase III, Beta Subunit
gp82(1)**	-
gp83	-
gp84(1)**	-
gp85(1)**	-
gp86	-

gp87	Ku Protein
gp88*	-
gp89*	-
gp90	ParB-like Protein
gp91	-
gp92*	-
gp93*	-
gp94	-
gp95*	-
gp96	-
gp97*	AAA ATPase
gp98	-
gp99*	-
gp100*	-
gp101**	-
gp102	-
gp103	-
gp104*	-
gp105*	-
gp106*	-
gp107*	-
gp108**	-
gp109	-
gp110	-
gp111	-
gp112(1)**	-
gp113*	-
gp114	-
gp115	-
gp116(1)**	-
gp117	-
gp118	-
gp119	-
gp120	-
gp121	-
gp122*	-
gp123	-
gp124	-
gp125	-
gp126	-
gp127	-
gp128**	-

Table 2. List of putative novel ORFs. Each potential novel ORF has one or more matching peptides. Shown are the genomic sequence regions of matching peptides along with the nucleotide length of each novel ORF using one or more predicted start codons.

Along with each possible start codon is the corresponding Shine-Dalgarno (SD) sequence with associated SD score based on the Kibler scoring matrix. See text for details. Note that all but one putative novel ORF has at least one possible associated start codon. Potential start codons are coded as ATG, GTG, or TTG.

Name	Start	Stop	Length	Start Codon	SD Score	SD Sequence
ORF-gp2609						
VLSRGLVL (763-786)	763	834	72	GTG	-6.167	GCCGCAGCCCGACACCAGTCG
ORF-gp2655						
WQPSSAFVM (6928-6954)	6862	6972	111	GTG	-6.299	TGCGCCACCTTCATGCACGAGG
	6901	6972	72	GTG	-4.884	TACCCCCAGAGCAAGGTCTCTG
ORF-gp2704						
RDVLGDAAR (13798-13824)	13399	13830	432	GTG	-5.462	TCGGCGTCAGCATCCCCCGCCA
	13441	13830	390	GTG	-5.733	TGACCGCGGTGCTGTTCGGGCT
ORF-gp2724						
GGLHPSV (18934-18954)	18580	19089	510	GTG	-6.530	GCGCGCAGAATCACGCGAAGGT
	18910	19089	180	GTG	-4.627	CCATCGACCGGACCCTCTCGCT
ORF-gp2754						
RAPTAGVR (25276-25299)	24892	25431	540	GTG	-5.338	CTCCAGGGCACGAATAGTCGGG
	24898	25431	534	TTG	-6.248	GGCACGAATAGTCGGGGTGGCC
	24928	25431	504	GTG	-9.061	TCGCCTTGCCGCCCTCCAGCGT
	25006	25431	426	GTG	-7.819	ACGGGCAGTCGTCTTCGATGGC
	25030	25431	402	GTG	-6.040	GGCCCACCCAGCTGCGCCCGAT
	25108	25431	324	TTG	-7.269	GTCGGCCTTCACCTGCGGCCTG
	25120	25431	312	GTG	-6.328	CTGCGGCCTGTTGCGCAGCACG
	25141	25431	291	GTG	-5.280	GGTGATCGCGGACTGCAGCGCG
	25144	25431	288	GTG	-5.452	GATCGCGGACTGCAGCGCGGTG
	25150	25431	282	TTG	-4.825	GGACTGCAGCGCGGTGGTGCGC
	25165	25431	267	ATG	-5.480	GGTGCGCTTGTCGGAGCCGGCC
	25180	25431	252	ATG	-4.150	GCCGGCCATGTTCAGGTTCTGG
	25210	25431	222	TTG	-5.043	CCCGGCCGACCGCAGGTCACGG
	25219	25431	213	GTG	-5.194	CCGCAGGTCACGGTTGGTTGGC
	25225	25431	207	ATG	-6.437	GTCACGGTTGGTTGGCGTGGTC
ORF-gp2836						
KRRSSPARSW (43288-43317)	43285	43446	162	GTG	-5.955	GTGTTCCCTGGCGAAACCCCCG
ORF-gp2907						
VGTIPSVER (53869-53895)	53824	54051	228	ATG	-7.827	CATCGACTCCACCACCGACGCC
	53848	54051	204	ATG	-4.782	GACCGGCAGCAGGCGCGGGTCG
	53866	54051	186	TTG	-7.897	GTCGATGTCGTCCTCGGTCACG
ORF-gp2974						
RLLVVGVE (63076-63099)	62725	63669	945	GTG	-6.240	CGCATGGCCAATCGACCTCGCA
	62803	63669	867	GTG	-5.942	TGCAGTTGCTCCACCGAGGGCG
	62854	63669	816	ATG	-4.966	CACGGGCGGCGGTGATCAGGTC
	62875	63669	795	GTG	-6.822	CATGGTTGGGCCTTGGGTAGTC
	62896	63669	774	TTG	-8.658	CGTGCACTTTCCTCATCGCCCG
	63055	63669	615	GTG	-6.866	CCGCCGACTCCAGCGCCGAAT

ORF-gp3257						
RCIGLSSSAR (40688-40717)	40565	40792	228	TTG	-6.434	GAGCTGTAACTCATGGCTGTCG
ORF-gp3260						
LCGCQRPG (41504-41527)	41480	41530	51	GTG	-6.003	TGTACGTCGGCAGCACAAAGGT
ORF-gp3338						
LPVQQLL (52229-52249)	52178	52510	333	GTG	-5.796	AGCGCGGTGACGACGACGAGAG
LPVQQLLL (52229-52252)	52235	52510	276	GTG	-5.531	TGATGGGGTTGTTGAGCTGCCG
ORF-gp3407						
HLIGAQR (66611-66631)	66521	66907	387	TTG	-8.432	GCCGATCATTCGCACCACCGCC
ORF-gp3525						
RSPVLAR (15777-15797)	15234	16079	846	GTG	-6.800	AGCCCGAGGCCCGCGCGTTGCC
	15252	16079	828	TTG	-5.380	TGCCGTGGATTCGACCGACAGC
	15288	16079	792	TTG	-5.709	CCGAGTCCTGGGCCACCCCGAG
	15456	16079	624	GTG	-4.820	CCGACCCCGAGGTCTACACCTC
	15570	16079	510	GTG	-7.479	CCGACCCCGAGGTCTACACCTC
ORF-gp3568						
RGGALVG (25137-25157)	25056	25307	252	GTG	-3.120	ATCACGGCCTCTTCAGGAACTC
ORF-gp3582						
QGVQRGAGLLR (32664-32696)	32577	32876	300	GTG	-6.494	TTCGGATCGGGTCCATTCCGGC
	32586	32876	291	GTG	-5.503	GGTCCATTCCGGCGTGGTCACC
ORF-gp3658						
QAVLAAHR (43848-43871)	43785	44282	498	GTG	-7.553	CCCGGCCGCTGTTTATCACCGT
	43806	44282	477	GTG	-4.235	TGTGCGGCACCGGTGTGCCCTG
ORF-gp3695						
VPVSIPRSA (50157-50183)	49917	50528	612	GTG	-6.346	ATAACCTGCTCGATGTACTTCT
	49932	50528	597	GTG	-7.373	TACTTCTGTGCGTCGGCAATTA
	49956	50528	573	TTG	-7.985	GTGCTCATCAAATCCTCCTGTA
	49965	50528	564	GTG	-6.648	AAATCCTCCTGTATTGTCGAAG
	50079	50528	450	ATG	-7.472	CTATCCGGCCACCCGCACCGGC
	50130	50528	399	TTG	-4.994	ATCCTGGAACACCACCGGCCTG
	50163	50528	366	GTG	-4.747	TCCGGCCATCAGCAACGTCCCG
ORF-gp3783						
AASVAAVL (67722-67745)	67599	68282	684	GTG	-7.322	CGGTCATCTTCCTTCTCGTGGA
	67716	68282	567	GTG	-6.398	AACCCACACCCTTGTCGGGGGT
ORF-gp5036						
LSNTLVT (22153-22133)	22159	22082	78	GTG	-6.968	AAGGAACTCCACGCAACGGCAGC
	22153	22082	72	TTG	-6.059	CTCCACGCAACGGCAGCGTGAAG
ORF-gp4983						
VAVPSAASF (29890-29864)	30121	29858	264	TTG	-6.666	GGCGGCGAGCGTCGCGACGAAC
	30118	29858	261	GTG	-5.888	GGCGAGCGTCGCACGAACTTG
	30115	29858	258	ATG	-5.423	GAGCGTCGCGACGAACTTGGTG
	30103	29858	246	ATG	-6.424	GAACTTGGTGATGTTCTCGGCG
	30088	29858	231	TTG	-7.024	CTCGGCGATGCCTGCGAGGCCC
	30082	29858	225	ATG	-5.955	GATGCCTGCGAGGCCCTTGCTG
	30052	29858	195	ATG	-6.695	GTCGGCGTCGAGCGCCGCACCC
	30013	29858	156	TTG	-7.545	GTCGGCGAACGTCTTGGTCTGC
	30004	29858	147	TTG	-7.101	GTCGGCGAACGTCTTGGTCTGC
	29926	29858	69	ATG	-4.711	CATCCAGGACCGCTCAGCCTGG
	29902	29858	45	ATG	-7.738	GACGTCGTACGCCGCCCGATTA

ORF-gp4617						
RVATAAV (17900-17880)	18347	17694	654	GTG	-6.884	CCGCGCGGTCCTCGGCGGTCAG
	18323	17694	630	GTG	-7.398	GCGCGCCGCGGCCACCCGACAG
	18314	17694	621	GTG	-3.018	GGCCACCCGACAGGTGATACAG
	18149	17694	456	GTG	-6.076	TGATGGCGCCGATGCTGCCGCG
	17903	17694	210	GTG	-5.770	TGTAGGCGGCGCTGCGGTTGAA
ORF-gp4598						
RPMPFTAVY (20984-20958)	21134	20898	237	ATG	-6.928	GCCGCTGCCCAGCGCCGCCGAC
	21104	20898	207	ATG	-3.618	CGGGATACCGGAGATCGAGCCC
	21056	20898	159	TTG	-6.245	CTCGAAGCCCGTCGAATGCGAG
	21047	20898	150	TTG	-6.063	CGTCGAATGCGAGTTGGTCGGG
	20999	20898	102	ATG	-5.199	GTCGCCCAGCACGTCCGGTGCG
	20993	20898	96	ATG	-4.290	CAGCACGTCCGGTGCGATGGCG
ORF-gp4595						
VLSLRLG (21494-21474)	22319	21264	1056	ATG	-4.959	ACATGGCTACCTCGGGAACAGA
	22292	21264	1029	GTG	-6.101	GTGTTCCAACAGCTTCTCGGCG
	22250	21264	987	GTG	-7.666	CGCAGCGACAACCTCCTCGACA
	22208	21264	945	GTG	-6.898	GAAGTCCGGCTTCACCTCGACT
	22199	21264	936	ATG	-6.535	CTTCACCTCGACTGTGATCGCC
	22121	21264	868	TTG	-6.131	CCCTGGTCACCGAGAACAGCGC
	21593	21264	690	GTG	-6.466	CGGCCTCGTCCTCGGTGTAACC
ORF-gp4594						
IGAQLAR (22412-22392)	22688	22341	348	GTG	-8.475	ACAGCACTCTCCCTCATCCGAT
	22634	22341	294	TTG	-6.731	GGCCAGCGAAGTCGTCGAAGAT
	22508	22341	168	TTG	-7.587	CCGGCAGCGTCGTTTTCCAGAT
	22454	22341	114	ATG	-6.174	TCTCCCGAAACGTCAGCGGCGG
ORF-gp4591						
DPVALAL (23279-23259)	23546	22788	759	ATG	-8.089	TTCCTTGCCCGCCACCTGCTGA
	23510	22788	723	GTG	-4.619	CAGCGGGTCGCCGGGGCGGCGC
	23357	22788	570	ATG	-7.775	CATCGTCCATCGTCCCGCCGAA
	23300	22788	513	GTG	-7.749	GAGTAGGCGTCTTCCAGCCCCA
ORF-gp4561						
LQRVGNL (30701-30681)	31850	30030	1821	ATG	-4.902	GTCGTCGTATGACCGGTAGGTC
LGADGGALR (31178-31152)	31838	30030	1809	TTG	-6.078	CCCGGTAGGTCATGTTCGGCTTC
	31835	30030	1806	TTG	-6.668	GTAGGTCATGTTCGGCTTCTTG
	31805	30030	1776	GTG	-6.945	GAAGAAGTACGCGCCGTGTGGG
	31802	30030	1773	ATG	-5.051	GAAGTACGCGCCGTGTGGGGTG
	31775	30030	1746	ATG	-5.996	GTTCGACGGGTACCGCTTCACG
	31772	30030	1743	TTG	-6.416	CGACGGGTACCGCTTCACGATG
	31598	30030	1569	GTG	-4.471	CAGGGGCGGGCCGGTGCCGGT
	31427	30030	1398	TTG	-6.001	CAATGCGGGTGATCCAATTCTC
	31064	30030	1035	GTG	-5.750	CAACCTGCCCGCCGGTCAGGCC
	30815	30030	786	GTG	-7.462	GGATCGACGCCGACTGCCGGGT
	30809	30030	780	GTG	-5.247	ACGCCGACTGCCGGGTGTGCAC
	30800	30030	771	GTG	-5.899	GCCGGGTGTGCACGTGGTTCTC
ORF-gp4470						
WLPSANHRRP (41147-41118)	41171	40953	219	GTG	-6.298	TCCTTAGTTCTTTGCCGGGTAG
	41159	40953	207	TTG	-5.428	TGCCGGGTAGGTGCGGCGGAAG
ORF-gp4462						
VLLRDAV (42422-42402)	42626	41916	711	GTG	-6.239	GGTAGGGGGTCACCGTGTTGAT

ORF-gp4461						
PSPTYSPPIR (43499-43470)	43544	42693	852	ATG	-4.263	GTAGCTGCGGAACTTCCTACGC
ASPRPSPTYSPPIR (43511-43470)	43532	42693	840	ATG	-5.259	CTTCCTACGCATGAAGTCGCGG
ASPRPSPTY (43511-43485)						
MRPISSLASPR (43532-43500)						
MRPISSL (43532-43512)						
ORF-gp4329						
SASAGRAVL (65369-65343)	N/A	N/A	N/A	N/A	N/A	N/A

Xenoreactive Immune Responses in Annelids:
A Flow Cytometric Study Investigating Natural Killer-like Activity of Earthworm Coelomocytes

Authors: Jennifer H. Daly, Shannon R. Cook, & Sheryl L. Fuller-Espie
Faculty Advisor: Sheryl L Fuller-Espie, PhD, DIC, Professor of Biology

This research was funded through the Cabrini College Biology Research Fund.

Abstract: Earlier studies by pioneers in the field of innate immune responses in invertebrates have investigated the capacity of earthworms (oligochaetes) to discriminate between self and non-self and mount an immune response in both allogeneic and xenogeneic pairings. Those studies used *in vivo* orthotopic allografts or xenografts of the body wall as well as *in vitro* cytotoxicity assays employing trypan blue uptake, lactate dehydrogenase release, and radioactive sodium chromate release protocols using isolated leukocytes (coelomocytes). Using two different genera of earthworms, our goal was to develop an *in vitro* flow cytometric assay to measure the natural killer-like immune response of *Lumbricus terrestris* when challenged by fluorescently-labeled coelomocytes of *Eisenia fetida*. Data is shown that demonstrates marginal, but significant, cytotoxicity at effector:target ratios of 5:1 and 1:1 following a two-hour incubation period compared to spontaneous cytotoxicity of control samples. These results, however, were not reproducible in other assays that were conducted using the same experimental conditions. We conclude that the mechanism by which graft rejection was operating in the earlier studies investigating graft rejection in earthworms was likely not exclusively due to natural killer-like cell killing, but instead also involves alternative necrotizing processes.

Introduction:

In comparison with the immune system of vertebrates, much less is known about the defense mechanisms employed by invertebrates. It has been strongly established, however, that invertebrates possess a robust innate immune system while vertebrates possess both innate and adaptive immune systems (reviewed in Bartl, Baish, Weissman, & Diza, 2003; Buchmann, 2014). Researching the innate immune responses employed by invertebrates to fight infection by pathogenic organisms is greatly important in the field of comparative immunology, especially from an evolutionarily standpoint (Rivero, 2006).

The cellular response of invertebrates includes natural killer-like activity, phagocytosis, encapsulation, nodule formation, coagulation, melanization and the production of toxic compounds. Pathogen-associated molecular patterns (PAMPs) trigger cellular activation of defense mechanisms while "self" molecules down-regulate cell activation of the innate immune response in order to prevent autoreactivity (Cerenius & Söderhäll, 2013). In addition, immune responses can be triggered following transplantation of foreign cells.

There are different types of transplantations including: 1) syngeneic transplantation which involves genetically identical or similar individuals of the same species; 2) allogeneic transplantation, which involves individuals that are of the same species but genetically different; and 3) xenogeneic transplantation, where individuals are genetically different and of different species. Xenotransplantation activates a wide variety of immunological responses. Rejection is a major obstacle that must be overcome by the immune system for successful transplantation. Due to a wide array of molecular incompatibilities between the host and donor, the innate immune response plays a large role in xenotransplantation (Wang & Yang, 2012).

In the 1960's Edwin Cooper conducted experiments on the earthworm response to *in vivo* orthotopic xenografting using the earthworms *Eisenia fetida* and *Lumbricus terrestris* from the family Lumbricidae (Cooper 1965a,b; Cooper 1968). The proposed role of earthworm coelomocytes in innate immunity can be observed when granular amoebocytes contact foreign target cells that are subsequently killed.

There are three different coelomocyte populations that are activated in the immune response of earthworms, these include, granular amoebocytes which possess the natural killer-like activity, hyaline amoebocytes which are phagocytic cells and dispose of the debris resulting from the destruction of target cells, and chloragocytes are thought to secrete humoral components (Engelmann, Cooper & Nemeth, 2005). Cooper's pioneering studies reported rejection of the orthotopic xenografts of integument.

In 1973 Cooper expanded on these studies by conducting *in vitro* experiments on the same earthworms to determine the response evoked with allogeneic and xenogeneic mixtures.

Two decades later, utilizing earthworm coelomocytes, Suzuki and Cooper (1995a, 1995b) were able to use a variety of *in vitro* techniques, including trypan blue exclusion, lactate dehydrogenase release and radioactive chromium-release assays. Suzuki and Cooper concluded that the index of viability decreased more rapidly in xenogeneic mixtures than allogeneic mixtures.

In an independent study involving sipunculids in addition to annelids, Valembois, Roch and Boiledieu (1980) sought to investigate cytotoxic capacity during allogeneic and xenogeneic graft rejection using a chromium-release assay. These studies included the xenografting and allografting of two different sipunculid worms, *Siphonosoma arcassonense* and *Sipunculus nudus*, and two different annelids, *Eisenia fetida andrei* and *Eisenia fetida fetida*. Through these studies it was observed that there is cytolysis in both xenografting and allografting. The xenografts showed cytolysis at a faster rate than allografts. They also reported that unlike sipunculids, annelids exhibited an enhanced second-set rejection response.

In 1995, Cooper et al. utilized earthworm coelomocytes in an experiment involving cytolytic activity against human tumor K562 cells (Cooper et al., 1995). Earthworm coelomocytes were used as effector cells against human tumor K562 target cells. It was observed that cytotoxicity was dependent upon the membrane adhering between small, electron dense coelomocytes and target cells. The authors concluded that the natural killer cell-like activity of recognition, binding, and killing of foreign cells is a characteristic of the natural immunity in earthworms.

In this study, we sought to determine if the graft rejection reported in the earlier orthotopic grafting experiments involving allogeneic and xenogeneic combinations of earthworms was indeed due to natural killer-like activity or if the rejection was attributed to other means. We conducted our assays using flow cytometry, a more sophisticated method that has not previously been used to study xenogeneic responses in earthworm coelomocytes *in vitro*.

Materials & Methods:

Harvesting of Coelomocytes

One day prior to extrusion of *E. fetida* and *L. terrestris*, a predetermined number of active healthy earthworms were selected from their habitats and placed, according to specie, into Petri dishes containing sterile paper towels saturated with 2.5µg/mL Fungizone (Fisher Scientific) overnight to reduce contaminants from the earthworm. The day following this overnight treatment, individual earthworms (both targets and effectors) were placed, using sterile forceps, into individual sterile troughs containing 3mL BD FACSFlow sheath fluid (BD Biosciences), the extrusion buffer where the earthworms would become agitated and release coelomocytes from the coelomic cavity through the dorsal pores.

The coelomic suspension was transferred into 0.5mL of Accumax™ (Innovative Cell Technologies, Inc.), an enzyme solution comprised of trypsin, collagenase, and DNAse, which was used to obtain single cell suspensions and disrupt cellular aggregates.

After a 5 minute incubation at room temperature, the cells were diluted with 5mL of PBS and centrifuged at 800 RPM (150g) for 5 minutes at 4°C. After decanting the supernatant, the cell pellet was resuspended in either 1mL of phosphate-buffered saline (PBS) (targets), or 1mL of BGS comprised of BaculoGold Media (BD Biosciences) supplemented with 10% fetal bovine serum, penicillin, streptomycin, amphotericin B, ampicillin, kanamycin, tetracycline, chloramphenicol, non-essential amino acids, HEPES buffer, and glutamine (effectors), as described in Cook, Sperratore and Fuller-Espie (2015).

A portion of the cell sample was then added to the hemacytometer and the cell concentration was determined using a phase contrast microscope.

Labelling Targets with DiO

Labelling of target cells was conducted using the LIVE/DEAD cell-mediated cytotoxicity kit from Life Technologies (catalog #L7010). The target cells were brought to the desired concentration by dividing the actual concentration of cells by the desired concentration, subtracting that number from the volume of media already used, giving the volume needed to be added to the sample in order to bring the cells to the desired concentration.

The specified amount of PBS was added to the cell suspension. Specified amounts of target cells to be labeled were transferred into sterile vials containing DiO (3,3'-dioctadecyloxacarbocyanine perchlorate) (2μL DiO/1× 10^6cells).

The cells were then incubated on a shaking incubator at 220 RPM, 25°C, for 20 minutes. The labeled target cell suspension was then transferred into a 15mL conical test tube, diluted with 7mL of PBS, and then centrifuged at 800 RPM, 4°C, for 5 minutes. The supernatant was then decanted, and the cell pellet was washed in 3mL of PBS and centrifuged at the aforementioned settings.

The supernatant was decanted and the cell pellet was resuspended in 1mL of BGS. The cells (10,000 – 25,000) were then added to a 96-well plate at 50μL/well.

Preparation of Effector Cells

The effector cells were brought to the desired concentration using the same method as the target cells using BGS. The effector cells (at effector:target [E:T] ratios as indicated in the results) were then added to the 96-well plate at 50μL/well.

Propidium Iodide

50μL of 75μM propidium iodide (PI) (provided in the kit) was added to treatment wells containing DiO-labeled targets and effectors.

Incubation & Flow Cytometry

After adding treatments and controls to all respective wells, the plates were placed into the centrifuge and spun at 800 RPM briefly. This process allowed the cells to settle to the bottom of the wells and facilitate physical contact between effector and target cells. The plates were then placed into the incubator for 2 hours at 25°C, 5% CO_2. After incubation, the contents of each well were transferred into flow cytometry tubes containing 50μL of ice cold FACSFlow. Samples were kept on ice in the dark (covered with foil) and run on the flow cytometer immediately. A minimum of 7500 events was collected for each sample.

Flow Cytometric Analysis of Cell-mediated Cytotoxity

A FACSCalibur flow cytometer (BD Biosciences) employing Cell Quest Pro Software™ was used for data collection without compensation. Final analysis used WinList™ 7.0 Interactive N-Color Compensation™ software (Verity Software House, Inc.).
Six controls were used in this assay including: unlabeled targets only; effectors only; unlabeled targets plus PI; DiO-labeled targets (green control); unlabeled targets plus PI and 50μL of 0.02% saponin (red control); and DiO-labeled targets plus PI (spontaneous death).

Following data acquisition, a sample containing only target cells was analyzed in order to create a region (R1) that included predominantly amoebocytes from *Eisenia fetida* (as shown in the upper left panel of Figure 1). A FL-1 (abscissa) versus cell number (ordinate) histogram gated on R1 was generated for the next step in the analysis (as shown in the upper right panel of Figure 1).

This gating strategy enabled the autofluorescent chloragocytes to be excluded from the final analysis. A second region (R2) was created in the FL-1 histogram to permit inclusion of only DiO-labeled target cells in the final analysis. In order to discriminate live from dead cells, a saponin-treated target sample (lower left panel of Figure 1) was included to facilitate the placement of the FL-3 quadrant marker (lower right panel of Figure 1).

The final analysis was gated on R1 and R2, and therefore included only targets cells. The number of targets that were killed by the effector cells exhibited dual staining with both DiO (green fluorescence) and PI (red fluorescence). Specifically, upon inspection of the lower right panel of Figure 1 the percent of dead cells is in the upper right quadrant of the FL-1 (abscissa) versus FL-3 (ordinate) histogram. This flow cytometric strategy is described in Patel, Francis, Cooper and Fuller-Espie (2007).

Results:

Four separate assays were conducted in triplicate using two different E:T ratios. Multiple assays were performed to demonstrate inter-assay reliability and reproducibility to observe the effects of xenoreactivity of the earthworm granular amoebocytes. Table 1 (assays 1, 3 and 4) and Table 2 (assay 2) depict the results showing the codification (identification number) of the *L. terrestris* and *E. fetida* used in each assay.

For example, assay 1 used effector cells from two different earthworms (Lt3 and Lt4), and target cells from two different earthworms (Ef5 and Ef12). Tables 1 and 2 also present the percentage of cells exhibiting spontaneous death (baseline) compared to death mediated by effector cells at the two different E:T ratios employed (percent death reflects DiO-labelled target cells [green] that are also PI-positive [red]).

Triplicates were averaged and the standard deviation was obtained. A student t test paired two sample for means was then used to determine the statistical significance (as depicted with asterisks, $p < 0.05$) between baseline versus treated target cells.

Results for assays 1, 3, and 4, shown in Table 1, indicate that in all but two cases (Assay 1 Lt4:Ef5 and Assay 4 Lt2:Ef10) cell killing was below baseline. Results for assay 2, shown in Table 2, show significant killing in all cases for at least one of the E:T ratios used. Figure 2 shows the data from Table 2 in bar graph format showing the error bars for standard deviation for three of the four samples at E:T ratios of 5:1 and 1:1. The Lt1:Ef3 data was not included in Figure 2 owing to the high level of spontaneous cell death observed with that particular target cell sample (80.33%).

There is a correlation between the degree of target cell killing and E:T ratio; the higher the ratio, the more killing occurred. In assay 2 a single effector cell population was used (Lt1) with four different target cell populations. In the other three assays, more than one effector cell population was used.

Discussion:

Cytotoxicity directed against xenogeneic targets was observed consistently in only one four of four assays conducted. In that assay, more target cell killing was observed when higher E:T ratios were used.

A relatively high level of spontaneous cell death was observed in DiO -labeled targets prior to exposure to effectors, perhaps attributed to sample preparation techniques. Although the results in assay 2 suggest that natural killer-like activity may be the underlying mechanism for graft rejection, this result was not reproducible in the other three assays carried out in this study.

Therefore, this study cannot conclusively state that natural killer-like cell death is the sole process responsible for graft rejection in this annelid model. Instead, cell death reported by Cooper in the *in vivo* orthotopic procedures may have been mediated by other causes such as insufficient vascularization or nutrient provision.

Future Directions:

These studies could be expanded to include a reversal of effector and target cells where *E. fetida* is the effector and *L. terrestris* is the target, and to employ different earthworm species such as *Eisenia hortensis*, an earthworm used regularly in our lab. In addition, implementation of longer incubation periods and higher E:T ratios may be necessary in order to observe consistent natural killer-like cytotoxicity in this *in vitro* model.

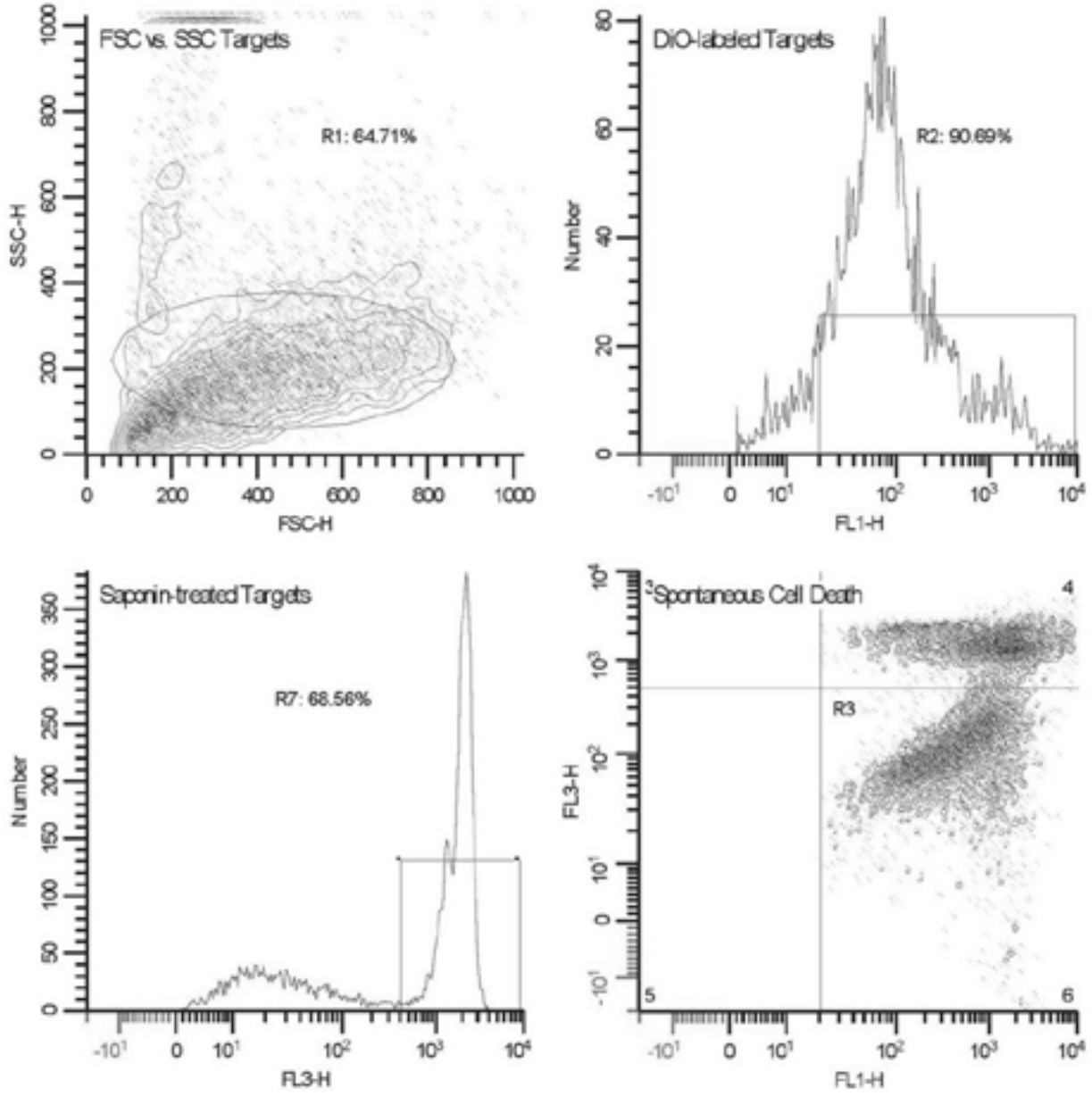


Figure 1: Depiction of the strategy employed to measure natural killer-like activity of *L. terrestris* effector cells when incubated with DiO-labeled *E. fetida* target cells. ***Upper left panel:*** Forward scatter (FSC-H) versus side scatter (SSC-H) histogram of DiO-labeled targets. Region 1 (R1) is drawn around the amoebocytes.

Upper right panel: FL-1 relative fluorescence intensity (RFI) versus cell number of DiO-labeled targets gated on R1. Region 2 (R2) is drawn around the cell population exhibiting high RFI in FL-1.

Lower left panel: Unlabeled targets treated with the detergent saponin (0.02%) and propidium iodide (PI) gated on R1. Region 7 (R7) depicts dead cells that have taken up PI. This control permits appropriate placement of quadrant markers in the lower right panel to distinguish dead cells.

Lower right panel: Fl-1 versus FL-3 histogram gated on R1 and R2 of target cells in the absence of effectors. The placement of the quadrant maker in the vertical position enables inclusion of only Di-O labeled cells, The placement of the quadrant marker in the horizontal position enables distinction between PI-positive (dead) versus PI-negative (live) cells. This control provides the spontaneous cell death baseline to which the E:T treatment samples are compared using student's t-test, paired two sample for means.

	Effector:Target E:T	Spontaneous Death (No Effectors)	E:T Ratio #1	E:T Ratio #2
Assay 1	Lt4:Ef5	56.31 (±2.29)	54.04 (±8.4)	60.71 (±0.37)*
	Lt3:Ef12	58.93 (±2.12)	55.30 (±9.06)	57.87 (±8.74)
Assay 3	Lt4:Ef3	54.38 (±0.73)	40.37 (±1.00)	45.70 (±1.10)
	Lt1:Ef5	67.39 (±1.82)	61.60 (±1.99)	64.81 (±1.03)
	Lt1:Ef6	62.81 (±0.67)	60.39 (±1.09)	62.65 (±0.59)
	Lt5:Ef7	64.64 (±1.00)	58.98 (±2.75)	62.04 (±1.32)
	Lt5:Ef11	37.57 (±0.98)	33.61 (±0.59)	32.16 (±0.33)
Assay 4	Lt5:Ef13	53.05 (±0.57)	48.39 (±0.52)	48.24 (±1.66)
	Lt18:Ef20	34.39 (±0.30)	33.94 (±0.04)	32.43 (±0.30)
	Lt2:Ef10	40.86 (±1.25)	40.82 (±4.34)	44.92 (±1.00)*
	Lt4:Ef12	18.92 (±1.74)	17.31 (±0.87)	18.23 (±0.23)

Table 1: Summary of % killing of DiO-labeled target cells in three independent assays. E:T ratio #1 was 5:1 for assay 1, 9:1 for assay 3, and 7.5: 1 for assay 4. E:T ratio #2 was 1:1 for assay 1, 1.8:1 for assay 3, and 1.5:1 for assay 4.
Values reflect % gated cells exhibiting dual fluorescence (i.e. DiO-positive and PI-positive), and standard deviations. Codification of individual *L. terrestris* (Lt) and *E. fetida* (Ef) earthworms is included. (* = $p \leq 0.05$)

Effector:Target (E:T)	Spontaneous Death (No Effectors)	E:T Ratio #1	E:T Ratio #2
Lt1:Ef3	80.33 (±0.58)	81.99 (±1.13)	82.31 (±1.10)*
Lt1:Ef8	48.61 (±1.17)	56.29 (±0.42)*	52.60 (±2.38)
Lt1:Ef10	44.47 (±0.86)	49.74 (±0.55)**	48.04 (±0.51)*
Lt1:Ef12	49.99 (±1.16)	52.77 (±0.87)*	50.81 (±0.17)

Table 2: Summary of % killing of DiO-labeled target cells in assay 2. E:T ratio #1 was 5:1 and E:T ratio #2 was 1:1. Values reflect % gated cells exhibiting dual fluorescence (i.e. DiO-positive and PI-positive), and standard deviations. Codification of individual *L. terrestris* (Lt) and *E. fetida* (Ef) earthworms is included. (* = $p \leq 0.05$; ** = $p \leq 0.005$)

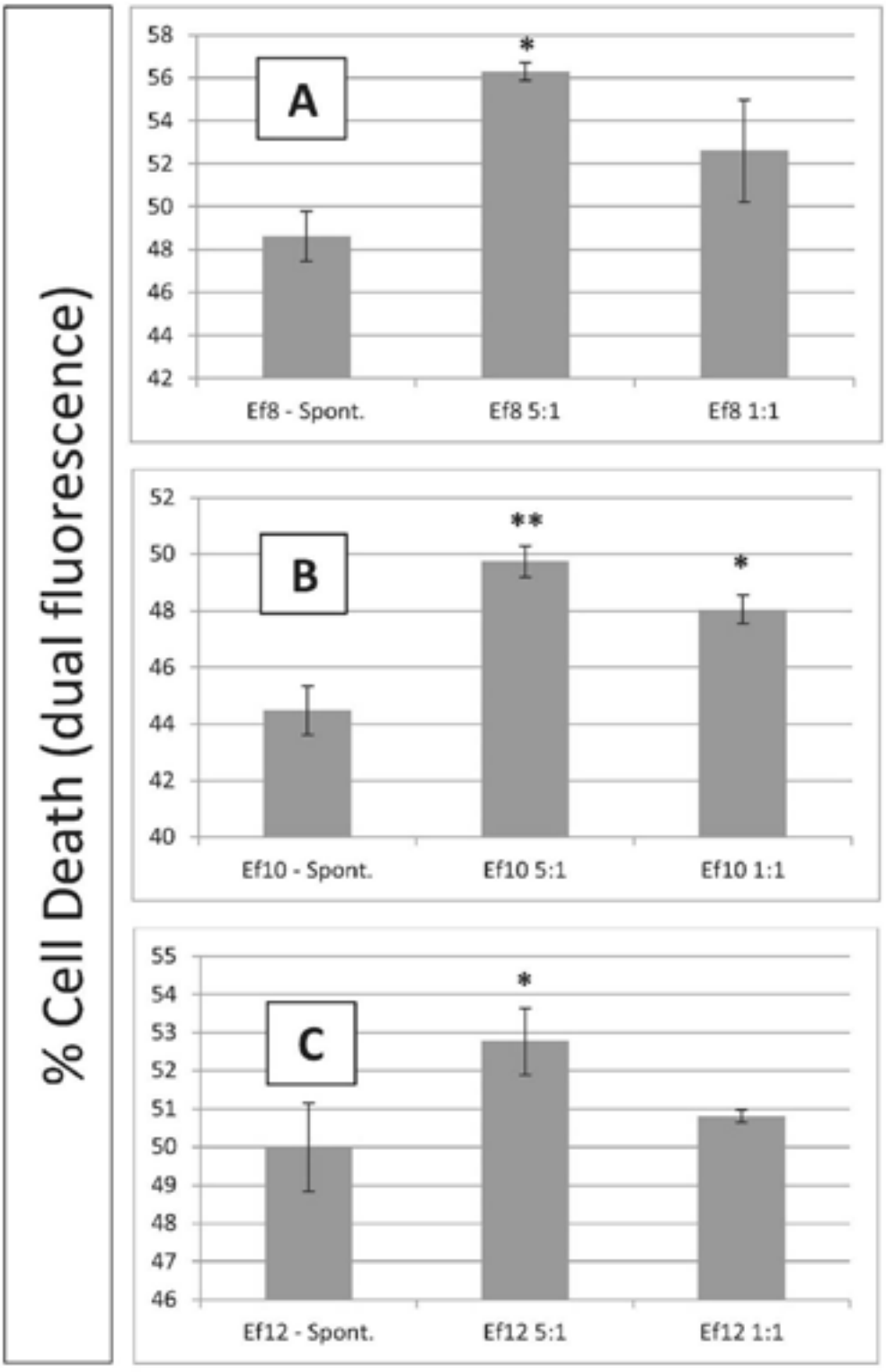


Figure 2: Percent killing of DiO-labeled target cells. Three different target cell populations of *E. fetida*, Ef8 (**A**), Ef10 (**B**), and Ef12 (**C**), at E:T ratios of 5:1 or 1:1 were incubated with the same *L. terrestris* effector cell population. (* = $p \leq 0.05$; ** = $p \leq 0.005$)

Bibliography:

Bartl, S., Baish, M., Weissman, I.L, & Diza, M. (2003) Did the molecules of adaptive immunity evolve from the innate immune system? *Integrative and Comparative Biology, 43*(2), 338-346.

Buchmann, K. (2014). Evolution of innate immunity: clues from invertebrates via fish to mammals. *Frontiers in Immunology, 5*, 459. doi: 10.3389/fimmu.2014.00459

Cerenius, L. & Söderhäll, K. (2013). Variable immune molecules in invertebrates. *Journal of Experimenal Biology, 216*, 4313-4319.

Cook, S.R., Sperratore, M.M., & Fuller-Espie, S.L. (2015) Nitric oxide production in celomocytes of the earthworm *Eisenia hortensis* following bacterial challenge. *Invertebrate Survival Journal, 12,* 46-65.

Cooper, E. L., Cossarizza, A., Suzuki, M. M., Salvioli, S., Capri, M., Quaglino, D., & Franceschi, C. (1995). Autogeneic but not allogeneic earthworm effector coelomocytes kill the mammalian tumor cell target K562. *Cellular Immunology*, *166*(1), 113–22.

Cooper, E.L. (1965a). Rejection of body wall xenografts exchanged *between Lumbricus terrestris* and *Eisenia foetida. American Zoology, 5,* 169-178.

Cooper, E.L. (1965b). A method of tissue grafting in the earthworm, *Lumbricus terrestis. American Zoology, 5,* 233.

Cooper, E.L. (1968). Transplantation immunity in annelids. I. rejection of xenografts exchanged between *Lumbricus terrestris* and *Eisenia foetida. Transplantation, 6,* 322-327.

Engelmann, P., Cooper, E.L., & Nemeth P. (2005). Anticipating innate immunity without a Toll. *Molecular Immunology, 42*, 931-942.

Patel, Francis, Cooper & Fuller-Espie (2007). Development of a flow cytometric, non-radioactive cytotoxicity assay in *Eisenia fetida*: An in vitro system designed to analyze immunosuppression of natural killer-like coelomocytes in response to 7, 12 dimethylbenz[a]anthracene (DMBA). *European Journal of Soil Biology, 43*, S97-S103.

Rivero, A. (2006). Nitric oxide: an antiparasitic moleculue of invertebrates. *Trends in Parasitology, 22*(5), 219-225.

Suzuki, M.M., and Cooper, E.L. (1995a). Spontaneous cytotoxic earthworm leukocytes kill K562 tumor cells. *Zoological Science 12,* 443-451.

Suzuki, M.M., and Cooper, E.L. (1995b). Allogeneic killing by earthworm effector cells. *Natural Immunity, 14*, 11-19.

Valembois, P., Roch, P, & Boiledieu, D. (1980). Natural and induced cytotoxicites in sipunculids and annelids. In *Phylogeny of Immunological Memory*. Edited by M.G. Manning, Elsevier – North Holland Biomedical Press, Amsterdam, pp. 47-55.

Wang, H., & Yang, Y.-G. (2012). Innate cellular immunity and xenotransplantation. *Current Opinion in Organ Transplantation*, *17*(2), 162–167.

ARTS • RESEARCH • SCHOLARSHIP SYMPOSIUM

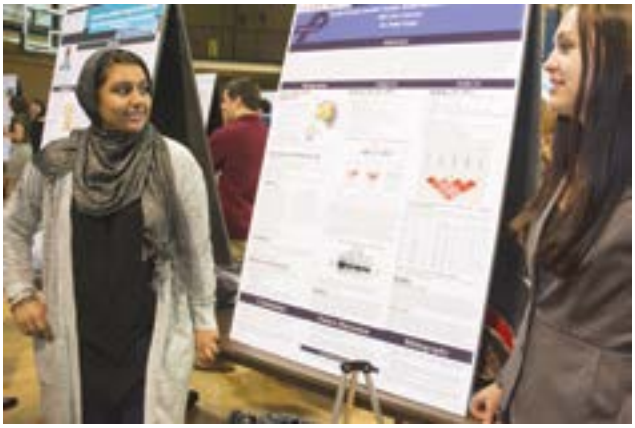
Cabrini celebrates its students' scholarly and creative accomplishments at the Arts, Research, and Scholarship Symposium, held each April.

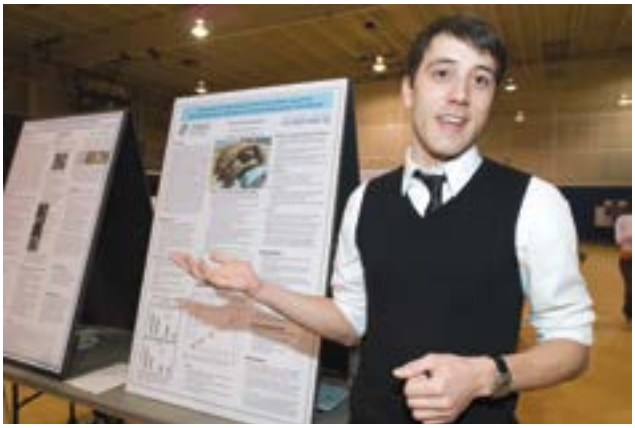
Students—undergraduate and graduate—are invited to present posters, artwork, oral presentations, and performances. On the day of the event, day classes are cancelled so the entire community can participate.

2016 marks Cabrini's tenth annual Symposium.

For more information, please visit **cabrini.edu/symposium**.

- Twitter: @CabriniSympos #CabriniSymposium
- Facebook: facebook.com/CabriniCollegeSymposium





CABRINI

610 KING OF PRUSSIA ROAD
RADNOR, PA

CABRINI.EDU