Preface

We’re excited to share the Summer Science Institute 2006 Journal. The Summer Science Institute began in 1990 supported by the Wisconsin Department of Public Instruction, the University of Wisconsin System, the UW-Madison Center for Biology Education, and the Howard Hughes Medical Institute. Four hundred and sixty-eight students have participated. Many of these are now beginning careers in science, engineering, medicine, and education.

The 2006 student participants and their faculty and staff mentors developed group research projects around several interesting scientific research problems and issues. These span several subdisciplines of biology. They include research on: ecology, aquatic biology, genetics, biotechnology, genomics, bioinformatics, immunology, neurobiology, physiology, neurophysiology, conservation & restoration, molecular biology and bacteriology.

The research articles were written by teams of students and were guided by their mentors. Summer Science Institute leaders and the mentors are proud of the work that you’ll find described in the student articles. What is not readily apparent, but merits saying is that the research reports that you see here resulted from hard work by all participants to meet many challenges. We especially want to acknowledge the personal and professional investment of the mentors in the students and the projects. We hope that this is just the beginning of the participants’ involvement in science.
Many thanks to the following for their support of our summer program and students

UW-Madison, Center for Biology Education (CBE)

Promega

The Biopharmaceutical Technology Center Institute (BTCI)

Institute for Cross-College Biology Education (ICBE)

Wisconsin Department of Public Instruction

UW System

UW-Madison, School of Education

Center for Instructional Materials and Computers (CIMC)

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Candidate Gene for ZYMV Resistance: eIF(iso)4E

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Abstract

Plant viruses cause a great deal of damage to many vegetable crops. One example of a devastating plant virus is zucchini yellow mosaic virus (ZYMV). The ZYMV infects many cucurbit species including cucumber and melon. Although pesticides have been used to control the virus, an ideal way to control the virus would be to have natural plant resistance in vegetable crops. Two candidate genes for ZYMV are eukaryotic initiation factor 4E (eIF4E) and eIF(iso)4E. A melon BAC containing eif4E has already been sequenced and we sequenced portions of a melon BAC containing eif(iso)4E to determine if the genomic regions surrounding the two genes were similar to indicate how long ago the genes duplicated. Comparison of the sequence between two BACs revealed only 50 bp of sequence similarity among the 25 kb compared. Therefore we determined that there is little similarity between the two genomic regions that these two genes diverged millions of years ago and that they may be responsible for different virus resistances.

Keywords
cucurbit, eif4E, eif(iso)4E, ZYMV (zucchini yellow mosaic virus), BAC (bacteria artificial chromosome)

Introduction

A survey conducted in Brazil from May of 1997 until June of 1999 showed that Zucchini Yellow Mosaic Virus (ZYMV) infects 24.8% of all cucurbit species (Yuki et al. 2000). ZYMV, also known as Muskmelon Yellow Stunt Virus, is a virus that causes severe damage to cucurbit plants around the world (Park et al. 2004). Cucurbits are plants in the family of Cucurbitaceae, which include cucumber, squash, pumpkin, gourds, watermelon and cantaloupe. The current method of halting the virus is to spray insecticide, which is undesirable due to the numerous problems that it causes. Other methods are expensive and are not viable solutions for stopping the virus. A new method of controlling the virus is needed. Breeding in characteristics that resist the virus is a more effective method of control.

Some things that may occur when a plant is infected with ZYMV are yellowing or mosaic pattern on the leaves, stunting of growth, reduction of seed production, fruit and/or seed deformation, and necrosis (Lecoq et al. 2002), which is the death of cells and tissues. Sap-sucking insects called aphids mainly transmit ZYMV. A possible method of controlling ZYMV is to spray insecticide on the plants to kill the aphids. However, it is environmentally undesirable and expensive to use insecticides. Historically, exposing the plants to the virus identified resistant plants, but inoculations are tedious and expensive. Resistances have been identified, but specific genetic causes are unknown.

Breeding plant resistance genes into the plant is the most cost effective and efficient method of controlling the virus. The eukaryotic initiation factor (iso) 4E (eIF(iso)4E) gene is a good candidate for virus resistance because the eIF4E and eIF(iso)4E genes are in all living things and bind to their RNA. However, they usually don’t bind to anything they don’t recognize, such as viral RNA. (Nicaise et al. 2003) The eIF4E gene has been proven to condition resistance to viruses in other plants, such as peppers (Ruffel et al. 2006) and lettuce (Nicaise et al. 2003). In melon, the eIF4E gene has already been sequenced and conditions resistance to Necrotic Spot Virus (Nieto et al. 2006). The goal of this research is to sequence the eIF(iso)4E gene and compare it to the sequence of the eIF4E to determine their differences and associate these differences with natural virus resistance. We think that eIF(iso)4E and eIF4E are good candidates for virus resistance because interaction with the viral RNA and eIF4E or eIF(iso)4E would allow the RNA to replicate and therefore cause infection. No interaction would cause the host to be resistant.

Materials and Methods

We received bacterial artificial chromosomes (BACs) containing melon DNA as stab cultures, or BACs in bacteria in a gel tube, from Clemson University Genomics Institute (CUGI). Next we grew the bacteria on agar plates containing Luria-Bertani (LB) broth and chloramphenical (34 µg/ml) by incubating at 37°C. Chloramphenical was important because it killed all the non-transformed bacteria, or bacteria that didn’t
contain a melon BAC. After that we grew the individual colonies in tubes, also with LB broth and chloramphenical at 37°C overnight in an incubator.

When the bacteria were ready we had to prepare them using solutions from the QIAgen Spin Prep Kit. To do this we harvested 1.5 ml of the culture into a 2 ml tube, spun it in a centrifuge, and decanted the liquid but left the remaining precipitate in the tube. We repeated this step to accumulate 3 ml of culture. Next we re-suspended the precipitate by adding 250 µl of re-suspension buffer solution (P1) and using a vortex mixer. We then added 250 µl of lysis buffer solution (P2) and inverted the tube 4-6 times to lyse the cells, which means break down the cell membrane. We separated the solid from the solution by adding 350 µl of neutralization buffer solution (N3) and inverting the tube 4-6 times. Then we placed the tubes on ice for 10-20 min. Next we centrifuged for 10 min at 4°C, transferred the liquid to a new tube and centrifuged for another 10 min. We then added 600 µl of isopropanol, mixed and let stand on ice for 5-10 min so the DNA would precipitate. To speed up the process, we put the tubes in a centrifuge at 4°C for 20 min. After that we removed the isopropanol and added 200 µl of wash buffer solution (PE). We centrifuged and let the tubes dry for 20-30 min. Then we added 20 µl of distilled water and incubated at 37°C for one hour. The BAC was now isolated.

We sub-cloned the BAC by using the TOPO Shotgun Sub-Cloning Kit according to the manufacturer’s protocol. Essentially, the BAC was split up into many small pieces and each was cloned into vectors creating new plasmids, which are double-stranded units of DNA that replicate within the cell without the chromosomal DNA. We took the plasmids and placed them into a tube with competent bacteria, bacteria able to include new DNA, so the bacteria could reproduce more clones of the plasmid. The newly transformed cells were incubated at 37°C in SOC media for one hour.

We plated the bacteria on agar plates containing ampicillin (50 µg/ml), LB growth media, and X-gal. Ampicillin selected against bacteria that haven’t incorporated the plasmid. X-gal caused bacteria carrying functional LacZ genes to turn blue. The insertion site for the sub clones is within the LacZ gene. Therefore, the colonies we used were not blue, because we wanted them to have insertions. We used the white colonies and grew them in 5.0 ml of LB growth media and 5 µl of ampicillin (15 mg/ml) overnight at 37°C. Once the cultures were ready we prepared them for sequencing using the QIAGen Spin Miniprep Kit according to manufacturer’s directions.

We set up sequencing reactions using primers M13 Reverse and T7. For the M13R reactions we added 4 µl of DNA from the plasmid preps, 2 µl of Big Dye, 3 µl of buffer, 0.75 µl of M13R primer and 10.25 µl of water to each PCR tube. For the T7 reactions we added 4 µl of DNA, 2 µl of Big Dye, 3 µl of buffer, 0.5 µl of T7 primer and 10.5 µl of water to each tube. Then we put the tubes in an Eppendorf Mastercycler Gradient Polymerase Chain Reaction (PCR) machine to run the sequence. We ran one cycle at 95°C for 2.5 min. Then we ran 50 cycles at 96°C for 25 sec, 55°C for 20 sec and 62°C for 5 min.

Once the sequencing was complete we had to clean it and remove unused reaction components. We did this by adding 20 µl of clean sequence beads (AGENCOURT) to each tube, which bind to the DNA directly. Then we added 80 µl of an 80% ethanol solution and mixed. We set the tubes in a magnetic plate for 2-3 min and the sequence beads containing the DNA were attracted to the sides in a circle. Then we withdrew the liquid and added 200 µl of 80% ethanol. We withdrew the liquid again and removed the tubes from the magnetic plate. We added 50 µl of water back to the tubes and mixed. When the beads were completely released from the sides of the tubes we transferred 10 µl of liquid from each tube to a plate for capillary sequencing and sealed it with foil. The University of Wisconsin-Madison Genetics and Biotechnology Center sequenced these using an ABI 3730 sequencer.

We downloaded the sequence data from the Biotechnology website (http://www.biotech.wisc.edu/) and analyzed by using a program called Sequencher 4.5. The program allowed us to view chromatograms, which are pictures that show the order of the base pairs in the DNA, of our sequence and look at the quality of it. We also used the Sequencher program to trim the ends and the vector. We determined which sequence would be used for alignment by utilizing the databases from the National Center for Biology Information. We assembled the sequence fragments into contigs (Figure 1). Then we corrected errors in the sequence. We uploaded the larger contigs along with the sequence from the elF(iso)4E BAC using NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST/). The results from the blast told us if there were any similarities.

**Results**

We completed 636 sequence reactions that generated 396 usable sequence fragments. We generated approximately 50,000 base pairs of genomic sequence from the melon BAC carrying elF(iso)4E. We produced 23 contigs and the largest has 15,550 base pairs of genomic sequence from the melon BAC carrying elF(iso)4E. We produced 23 contigs and the largest has 15,550 base pairs (Table 1). Only the three largest contigs, and the contig containing the elF(iso)4E gene were used for further analysis.

We compared our data to the sequence of the elF(iso)4E BAC using the NCBI database. We blasted contig 3 and there were similarities to *Cucumis sativus* (cucumber) CsRP1 repeat region sequence. Contig 7 shared similarities with *Cucumis melo* (melon) truncated ACC synthase gene. Contig 12 was
Figure 1. Contiguous sequence (Contig) developed from assembling random sequence fragments. This is a picture of contig 7. It is comprised of 20 DNA fragments and is 5000 based pairs long.

similar to *Arabidopsis thaliana* GTP binding (AT1G52980) mRNA. Contig 16 was similar to *Capsium annuum* (pepper) cultivar DH801 eukaryotic initiation factor 4E (eIF(iso)4E) mRNA, *Nicotiana tabacum* (tobacco) eukaryotic initiation factor iso4E mRNA, and *Pisum sativum* (pea) cultivar JI2009 eukaryotic translation initiation factor 4E isoform (eIF(iso)4E) mRNA. When we compared these contigs, the only contig that shared any similarity to the eIF4E BAC was the contig carrying the eIF(iso)4E gene. It had 51 matching base pairs and was 88% similar to the eIF4E gene.

**Discussion**

The genes eIF4E and eIF(iso)4E are the result of gene duplication in plants. Gene sequences remain similar because they are essential for plant function. One fragment of the eIF4E and eIF(iso)4E genes in melon was 88% similar (Figure 2). However, our research has shown that the genomic regions around eIF4E and eIF(iso)4E are not similar. Database searches revealed no sequence similarities for the comparison of sequence surrounding the eIF(iso)4E gene to the sequences surrounding eIF4E. We conclude that this sequence is non-coding and diversified over time. Therefore, the duplication giving rise to eIF4E and eIF(iso)4E must have occurred so many years ago that there is no longer any detectable similarity among flanking regions.

The next step would be to associate eIF4E and eIF(iso)4E with virus resistance and susceptibility. To do this, sequences must be obtained from plants that are resistant and from plants that are resistant and from plants that are susceptible. These

Table 1. Contigs

This is a table of all the contigs we produced from our DNA fragments. The first column is a list of all the contigs we have. The second column is the number of base pairs that each contig has and the third column is the number of sequence fragments in each contig. Contigs with an asterisk next to them are the contigs that we used for comparison to the sequence from the eIF4E BAC. We currently have 23 contigs from 336 DNA fragments that make up approximately 50 kilo bases.

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*denotes contigs that were used for further analysis.
sequences will then be compared to each other and the sequences we already have of the two genes. If a specific sequence is always obtained from resistant plants and if the different sequence is always found in susceptible plants, then it may mean that eIF4E or eIF(iso)4E condition resistance

Conclusion

- eIF4E and eIF(iso)4E are similar, but surrounding genomic regions are dissimilar.
- These genes duplicated a long time ago.
- We can use these sequence differences to associate eIF4E and eIF(iso)4E with virus resistance and susceptibility.

Acknowledgements

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Literature Cited


**“Is it On Yet: Rubisco Gene Expression in Tomato Seedlings”**

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**Abstract**

Gene expression is a measure of when a gene is OFF or ON. It is important during such critical life processes as development, cell division, germination, and photosynthesis. In this experiment, we analyzed expression of Ribulose-1, 5-bisphosphate carboxylase oxygenase, a gene involved in photosynthesis. To determine when the Rubisco gene is turned on during development and where in a plant Rubisco is turned on, we analyzed tomato seedlings by isolating RNA and using RT-PCR. We learned that the Rubisco gene is expressed as early as Day 3, that Rubisco gene expression can be increased by light by Day 5, and that Rubisco is on in leaves, stems, and roots by Day 12.

**Introduction**

Have you ever wondered why your hair color may be different from that of someone in your family? The reason why you may have different characteristics such as eye color, hair color, or skin type is because that gene is embedded into your DNA. Every organism on earth has genes, and some, such as plants, can even regulate when their genes are expressed. An example of this is the regulation of photosynthesis in plants, since the plants regulate when photosynthetic genes, such as Ribulose-1, 5-bisphosphate carboxylase oxygenase, are turned on or off.

Deoxyribonucleic acid (DNA) contains the code for you, me, and everything living. DNA is a nucleic acid that carries the genetic information in the cell (see Figure 1). DNA is made of four nucleotides called Adenine, Thymine, Cytosine, and Guanine (A, T, C, and G). The sequence of nucleotides determines individual hereditary characteristics such as eye color, hair color, and blood type. In plants, this can determine traits such as leaf shape, color, and height of the plant. These trait encoding sequences are called genes (see Figure 2). If even a single nucleotide in a sequence is disrupted, the trait which is formed by that sequence would change. Although DNA can do so much for the traits of an organism, it just would not be the same without genes. Genes are hereditary units that are encoded in the DNA of the organism. All living organisms are built up of numerous genes, however, not every gene is active or always on.

Information within a gene must be expressed as a protein (See Figure 3). The process in which genes are changed into proteins consists of DNA being transcribed into RNA, and from RNA it is translated into protein. This protein that is formed results in a specific trait. The process of turning on and off different genes is called gene regulation. When genes are irregularly turned on or off, they cause a change in traits. An example of the effects of genes improperly being turned on or off would be cancer. Cancer is caused by mutated genes that regulate the behavior and growth of a cell. Before these genes are mutated they would usually limit the production of cells and tell the cells when to repair DNA damage, or failing that, to self-destruct - a process called apoptosis, or cell 'suicide’ (“Oral Cancer”). Humans have the ability to regulate genes during embryonic development to form arms, legs, and body organs.
Plants can also regulate genes during two important processes—germination and photosynthesis (See Figure 4). In order for a seedling to develop into a plant, it first has to be germinated. Seed germination requires water, oxygen, and a temperature of about 70 degrees Fahrenheit at best (Hudson). By placing the seed in a moist place where there is ample oxygen and a pleasant temperature, the seed will begin to germinate into an adult plant.

**Requirements for Germination**
- Water (for moisture)
- Oxygen
- Temperature

**Requirements for Photosynthesis**
- Chloroplasts
- Light absorbing pigments
- Light Waves
- Water
- Carbon Dioxide
- Oxidation/Reduction Molecules

Photosynthesis is the process by which plants, bacteria, and some protists use the energy from sunlight to produce sugar. Afterward, sugar is then converted to ATP during cell respiration, which cellular respiration converts into ATP (the "fuel" used by all living things). An example of a gene whose expression is regulated during photosynthesis is the (Rubisco) enzyme. Rubisco is the world’s most abundant enzyme and plays the largest role in photosynthesis. It makes up about 50% of the leaves protein content (Bonner and Varner, 1976; Ellis, 1979). Rubisco is used to fix CO₂ during the photosynthetic process (Campbell, et. al). There are many different types of Rubisco such as the small subunit (RbcS) and large subunit (RbcL). Expression of both genes is required for a functional enzyme (Bourque, Coen, McIntosh).

The expression of the Rubisco gene is dependent on a variety of factors including levels of CO₂ (Pandurangam, V et. al., 2006), temperature (Zhou, Y.H. et. al. 2006), and light (Tobin and Silverthorne, 1985). For example, when light is present, the Rubisco gene is typically turned on, and when light is not present, the Rubisco gene is not active. The reason is that Rubisco is an enzyme that is only needed during photosynthesis. Photosynthesis requires light, hence when light is present, the Rubisco gene is expressed. In addition, Rubisco gene expression is also organ-dependent. In a paper by Coruzzi et al. (1984), Rubisco has been found to be present in leaves, stems, pericarps, petals, and seeds of peas. Although roots have been tested for Rubisco, the gene was not expressed in that particular tissue. These pea plants that were tested were seven days old before being harvested for testing. Light also had an effect on Rubisco gene expression. Rubisco gene expression has also been studied by SSI groups in 2004 and 2005. In 2004, the SSI group found that mature tomato leaves turn off Rubisco genes after prolonged exposure to the dark, and turn the Rubisco gene on only 2 minutes after re-exposure to light (Avalos, et. al). In 2005 the group found that red light may be the best type of light for Rubisco gene expression. That study focused on whether different light colors affected the expression of the Rubisco gene. The result of that study was that Rubisco is always turned on in mature tomato leaves (Beben Y., et. al.).
Figure 5A. Molecular model of Rubisco.
http://www.photosynthesisresearch.org/images/Andersson%20rub3L.jpg

Figure 5B. Role of the Rubisco enzyme in photosynthesis

Last year's study relates to our hypothesis because we then wanted to know if in beans, the development and light would affect the Rubisco gene expression. Therefore, we hypothesized that in beans, if Rubisco is a gene involved in photosynthesis and plant embryos have not started photosynthesis, the Rubisco gene will not be active in plant embryos. To test this hypothesis, tomato seeds were grown in light and dark conditions. At different stages of growth, the total RNA was isolated and an RT-PCR was used to specifically detect the Rubisco RNA.

By detecting the first signs of a gene, it helps further the scientific knowledge of development in living organisms. By gaining more knowledge of detecting the Rubisco gene translation in the early stages before development of the actual gene that helps photosynthesis in plants, we will be able to gain more knowledge into the actual development of plants which can provide a strong basis for other scientists studying the many stages of cell development in organisms.

Materials and Methods

Growing Tomato and Bean Plants
Beans (Blue Lake 274) and Tomato (Jetsetter Hybrid) seeds were purchased from J.W. Jung Seed Co. (Randolph, WI). To germinate these seeds, we placed them on moist paper towels inside the Petri dishes. We then gathered the dishes of the seeds that will be placed in the light and put them into a small Tupperware container that had water to keep the paper towels moist. Then we did the same for the dark samples and placed it inside a box that was closed up with a small entry way on the side. The box was placed inside a cabinet in the dark room. The temperature in both light and dark conditions was 20˚C. To observe plants and to harvest the plants in the dark, we used a green light. We used a green light because plants don’t absorb the color green like they would if we would have used a red, blue or any white light to start the process of photosynthesis and potentially turn on the Rubisco gene.

Isolating RNA from Tomato and Bean seedlings
We followed a process to isolate the RNA using materials provided by the SV RNA Total Isolation System (Promega; Madison, WI). Whole seedlings from the same growth stage or roots, stems and leaves were placed in a mortar and crushed into a fine powder with a pestle in the presence of liquid nitrogen. The liquid nitrogen was important to freeze the cells, keeping them from performing photosynthesis. Once the plant material was crushed into a fine powder, approximately 100 mg of the sampling was placed into 1.5 ml microcentrifuge tubes and 583 µl of lysis buffer. For every extra milligram of sample, 5.83 µl of lysis buffer was added. Then, 350 µl of the RNA dilution buffer was added to the tubes and the solution was mixed by inverting 3-4 times. The tubes were then placed in an Eppendorf centrifuge 5415C (Hamburg, Germany) for 10 minutes. After 10 minutes, the clear lysate solution was transferred to another microcentrifuge tube using a micropipette (avoiding the pelleted debris). Next 200 µl of 95% ethanol was added to the lysate solution, and then the solution was mixed by pipetting 4 times. The samples placed
1 day old tomatoes
- No germination but swollen
- No roots
- No stems
- No leaves
- Brownish color

3 day old tomatoes
- Most have 2 mm roots
- No stems
- No leaves
- Brownish color

4 day old tomatoes
- Most have roots about ¼ of the size
- Starting to have stems ¼ mm long
- No leaves
- Brownish color

5 day old tomatoes
- Roots are about ½ the size of actual tomato seedling
- Growing stems about 1mm long
- Have started producing chlorophyll
- No leaves
- Brownish color

6 day old tomatoes
- Roots are about ½ inches long
- Stems are about 2-3mm long
- Few have small leaves
- Brownish/greenish type color

9 day old tomatoes
- Roots are about 1 ½ inches long
- Stems are about 5mm long
- More are producing small leaves
- Greenish/brownish type color

12 day old tomatoes
- Roots are about 2 inches long
- Stems are about 8mm long
- Bigger leaves are growing

Figure 6. Observations of Tomatoes during Days 1-12 after planting

Rubisco mRNA was analyzed by Reverse Transcriptase-Polymerase Chain Reaction using the Access RT-PCR System Kit (Promega, Madison, WI). First a master mix was prepared. The master mix contains appropriate multiples of the following: 32 µl of nuclease-free water, 10 µl of the AMV/Tfl 5X Reaction Buffer, 1 µl of the dNTP Mix (nucleotides), 1 µl of the downstream primer (sequence 5'-GCTTGTAAGCGATGAAGCTGATGCACTGC-3'), 1 µl of the upstream primer (sequence 5'-CCCTTCACTGGACTCAAGTCCACCGC-3'), 2 µl of the 25 mM MgSO4 and, 1 µl of the Tfl DNA Polymerase. Then 48 µl of this master mix solution was distributed to each of the RNA samples and the negative control sample. The positive control was set up according to the directions provided by the Access RT-PCR System Kit, which used different primers. Then, 1 µl of the AMV reverse transcriptase was added to the RNA samples. 1 µl of the RNA was added to each reaction except the negative control-1 µl of the nuclease-free water was substituted for the RNA. Tomato leaf RNA known to contain Rubisco mRNA was used as a positive control. All of the samples and controls were placed in thin-walled 0.5 ml reaction ‘Thermowell’ tubes (Corning Inc.; Corning NY) and

Testing RNA Amount, Purity, and Integrity
The RNA samples were analyzed using the Milton Roy spectronics 601 spectrophotometer (Ivyland, PA) to determine RNA mass and contamination numbers. 50 µl were taken from the RNA samples and mixed with 950 µl of nuclease-free water for analysis. The wavelengths were set at 260, 280 and 230 nanometers for RNA, protein, and guanine thiocyanate (GTC) contents respectively. The actual mass of RNA was found by taking the A260 reading and multiplying it by 40 µg/ml * dilution factor of 20 (RNA sample/RNA + nuclease-free water) * 0.1 liters. The protein and GTC contamination was analyzed as a ratio of A260/contaminant reading.

The RNA samples were also analyzed for integrity by running the RNA through a 1.5% Agarose (Promega; Madison, WI) gel electrophoresis. 10 µl of RNA sample and 5 µl of RNA loading dye were loaded into the wells. In the first well, 5 µl of RNA 100 base-pair ladder marker was loaded. The gel was run at 150 volts for 30 minutes. The gel was analyzed for the presence of two bands, 28s and 18s ribosomal RNA.

RT-PCR and Gel Electrophoresis
Presence of Rubisco mRNA was analyzed by Reverse Transcription-Polymerase Chain Reaction using the Access RT-PCR System Kit (Promega, Madison, WI). First a master mix was prepared. The master mix contains appropriate multiples of the following: 32 µl of nuclease-free water, 10 µl of the AMV/Tfl 5X Reaction Buffer, 1 µl of the dNTP Mix (nucleotides), 1 µl of the downstream primer (sequence 5'-GCTTGTAAGCGATGAAGCTGATGCACTGC-3'), 1 µl of the upstream primer (sequence 5'-CCCTTCACTGGACTCAAGTCCACCGC-3'), 2 µl of the 25 mM MgSO4 and, 1 µl of the Tfl DNA Polymerase. Then 48 µl of this master mix solution was distributed to each of the RNA samples and the negative control sample. The positive control was set up according to the directions provided by the Access RT-PCR System Kit, which used different primers. Then, 1 µl of the AMV reverse transcriptase was added to the RNA samples. 1 µl of the RNA was added to each reaction except the negative control-1 µl of the nuclease-free water was substituted for the RNA. Tomato leaf RNA known to contain Rubisco mRNA was used as a positive control. All of the samples and controls were placed in thin-walled 0.5 ml reaction ‘Thermowell’ tubes (Corning Inc.; Corning NY) and

into a spin basket (a tube within a 1.5ml collection tube which has a filter of positive charge at the bottom and centrifuged at 14000x g for one minute. The liquid in the collection tube was discarded, and then 600 µl of SV RNA wash solution was added to the spin basket. The tubes were placed back into the centrifuge for one minute. The collection tube was emptied and 50 µl of freshly prepared DNAse incubation mix was distributed to each sample. The DNAse incubation mix consisted of 40 µl of yellow core buffer, 5 µl MnCl2, and 5 µl of DNAse. The samples were incubated for 15 minutes at room temperature. Next 200 µl of the SV RNA wash solution was added and centrifuged for one minute. Finally, 250 µl of the SV RNA wash solution was added and centrifuged for 2 minutes. The spin basket was then placed in a 1.5ml elution tube provided by the SV RNA isolation kit and 100 µl of nuclease free water was added. The tubes were centrifuged for one minute. Afterwards, the spin basket was discarded, and the elution tubes that contained the purified RNA were capped and stored in a -70°C freezer (Forma, Nepean, Ontario, Canada).
then spun down for 10 seconds. Two drops of nuclease-free water mineral oil were then added in order to weigh down the reaction mixture. The reaction tubes were then placed inside the Perkin Elmer DNA Thermalcycler 480 (Richfield, NJ) for the cDNA synthesis and amplification to occur.

The thermocycling steps for the first strand cDNA synthesis occurred as following: 1 cycle of 48 degrees for 45 minutes (reverse transcription), and then 1 cycle of 94 degrees for 2 minutes (AMV RT inactivation and RNA/cDNA /primer denaturation). The thermocycling steps for the second strand of cDNA synthesis and PCR Amplification occurred as follows: 40 cycles of 94 degrees for 30 seconds (denaturing), 60 degrees for 1 minute (annealing) and 68 degrees for 2 minutes (extension); 1 cycle of 68 degrees for 7 minutes (final extension) and 1 cycle of 4˚C (soak).

The samples were then analyzed by a 1.5 Agarose Gel Electrophoresis and stained with 0.5 ul ethidium bromide/ 10 ml TBE. Tubes were prepared with 10 µl of the RT-PCR and 2 µl of blue-orange loading dye (Promega; Madison, WI). 5 µl of the 100 base pair ladder (Promega; Madison, WI) was loaded into the first lane; the subsequent lanes were loaded with 11 µl of the RT-PCR samples and the control samples. The gel was run at 150 volts for about 30 minutes; the gel was analyzed under UV light.

**Results**

**Observations of Plant Growth**

After we planted our seedlings, we observed their growth each day. According to our observations the first sign of plant growth was on day 3 when it started to grow roots. The stems didn’t start to grow until about day 4 and the chlorophyll didn’t start to show until about day 5. In the dark, the development was slower. It showed that the leaves, stems and roots were growing just not at the same rate as the ones in the light. After observing the seedlings in the dark it was concluded that they were not producing any chlorophyll.

**UV Spectrophotometer Analysis of Isolated RNA**

We used UV Spectrophotometry to determine how much total RNA was isolated from our plant samples. In figure 7A, we learned that we were able to isolate 0.12 µg RNA/ mg Sample. This was a 71% increase from last year’s data analysis.

In Figure 7B, we compared the average RNA yield from leaves versus embryos, bean leaves versus tomato leaves, bean embryos versus tomato embryos, and plants that were kept in the light to those that were kept in the dark. There appears to be more RNA present in the embryos in comparison to plants with mature leaves. There is no difference in the amount of RNA isolated from bean leaves and tomato leaves. However, these results show that there are higher readings of isolated

<table>
<thead>
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<th>Sample Type</th>
<th>Average µg RNA/ mg sample</th>
<th># of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEAF</td>
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<td>11</td>
</tr>
<tr>
<td>EMBRYO</td>
<td>0.15</td>
<td>27</td>
</tr>
<tr>
<td>BEAN LEAF</td>
<td>0.10</td>
<td>6</td>
</tr>
<tr>
<td>TOMATO LEAF</td>
<td>0.09</td>
<td>5</td>
</tr>
<tr>
<td>BEAN EMBRYO</td>
<td>0.19</td>
<td>17</td>
</tr>
<tr>
<td>TOMATO EMBRYO</td>
<td>0.09</td>
<td>11</td>
</tr>
<tr>
<td>LIGHT</td>
<td>0.18</td>
<td>28</td>
</tr>
<tr>
<td>DARK</td>
<td>0.04</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 7. UV Spectrophotometer Analysis of RNA Isolated from Beans and Tomatoes.

RNA in plants that were left in the light than those that were left in the dark. This is also shown in the results from gel electrophoresis tests. It is also shown that more RNA was isolated from the embryonic stages in bean plants than from bean leaves. But this is not also the case with the tomato leaves. It is shown that the averages were equal which is also determined on the number of samples that we tested. But in comparison bean leaves had shown more isolated RNA than leaves of tomato plants and also that bean embryos also had more RNA to be isolated in them than tomato embryos.

In data not shown the average GTC contamination was “undefined”. However, there were some GTC measurements which were based on zero or negative A230 spectrophotometer readings. We felt that these data points were inaccurate. This did not allow us to use all A230 data points to determine the average level of GTC contamination. Without these confounding points, the average GTC contamination was 0.37.

**RNA Gel Electrophoresis**

A RNA gel was prepared to analyze the purity of the isolated RNA (figure 8). This gel shows the 28s and 18s are in a 2:1 ratio, implying that the RNA is intact and not degraded. These results were positive because degraded RNA can not be used
in a RT-PCR to detect the Rubisco gene. From analyzing this gel it is feasible to say that our RNA was intact and useable.

![Figure 8](image1.png)

**Figure 8.** Gel Electrophoresis of total RNA from mature bean and tomato leaves.

### 1,3,5,7 Light vs Dark

To determine when the Rubisco gene is turned on during development and to see whether or not Rubisco gene expression is dependent on light, we isolated RNA from tomato seedlings in light and dark one, three, five and seven days after “planting”. The presence of Rubisco RNA was determined by RT-PCR (Figure 9). This gel shows that in the light, there is a steady increase of Rubisco RNA from Day one through Day seven with the Rubisco gene turned on by Day 3. In plants grown in the dark, the Rubisco gene is also turned on by Day 3, however, the amount Rubisco does not increase. In fact, there is a decrease in the amount of Rubisco RNA present by Day 7. Overall, Rubisco gene expression begins early in seedling development and responds to light.

![Figure 9](image2.png)

**Figure 9.** RT-PCR Analysis of RNA isolated from Tomato

### RT-PCR Analysis of Rubisco Gene Expression in leaves, stems, and roots, of 12 day old tomato seedlings

To determine which plant parts produce Rubisco RNA total RNA was isolated from leaves, stems, and roots of 12 day old tomato seedlings. RT-PCR was used to specifically detect the presence of Rubisco RNA and the results were analyzed by gel electrophoresis (Figure 10). Among analyzing the data, Rubisco seemed to be active (on) in the leaves of the light plant, slightly on in the stem of the light plant, and on in the roots in the light plant, yet the leaves produced a more intense band which indicates that the Rubisco was more present in the leaves. In the dark the leaves were on again yet the bands weren’t as dense as those in the light. It was slightly on in both the stem and root of the plants germinated in the dark.

Based on these results it is evident that light seems to effect the expression of Rubisco in leaves and roots and that Rubisco is expressed in all three plant tissues.

![Figure 10](image3.png)

**Figure 10.** Rubisco gene expression in leaves, stems, and roots of 12 day old tomato seedlings.

### Discussion

**Total cellular RNA is dependent on many factors**

Spectrophotometer analysis of our isolated RNA shows that total cellular RNA is dependent on many factors. One factor is that embryos may have 50% more RNA than mature leaves (see figure 7). These may be because embryos are just beginning to grow roots, stems, leaves, and develop other features. During development a higher amount of RNA is being transcribed into proteins that then have to combine into like groups to actually form organ tissue and all the necessary organs for an organism to grow properly and to survive.

Another factor which may influence total cellular RNA is plant type. Our results also show that bean embryos produce more than double the RNA produced by tomato embryos. Although this observation is interesting we do not have an adequate explanation. Further research is needed.
A final factor that we observed that may influence total cellular RNA is light exposure. There was over 400% more RNA isolated in embryos grown in the light than in the dark. This implies that many genes in plants might be light regulated. This would make sense since plants would not waste energy transcribing genes when they are not needed.

Rubisco gene expression is regulated by development and light.

Based on our RT-PCR analysis of tomato seedling RNA, we found that Rubisco could be detected in tomato embryos (see figure 9) by day three very young stage. Others have also detected Rubisco RNA at an early stage, however our research shows that Rubisco RNA is present even earlier than that. This maybe because of the methods used to detect Rubisco RNA. This group used radioactivity where as we used RT-PCR which is an even more sensitive method of RNA detection. Furthermore, our results show that light is not a factor for the initial turning on of the Rubisco gene since Rubisco RNA was also detected in three day old tomato seedlings grown in the dark. The Rubisco RNA could be detected in both dark and light grown tomatoes by age day three. So we concluded that light is not needed to turn on the rubisco gene. We think this is so because seeds are usually planted underground where there is no light present. By having the Rubisco gene on even in the dark, the plant is prepared for photosynthesis at the first exposure to light.

Our results show that the Rubisco gene expression increases by day five in response to light. At this stage of new development, chlorophyll has also become present. Chlorophyll is an essential part of photosynthesis since it absorbs the light energy necessary for the reaction to work, because the plant can now perform photosynthesis. Their may be some positive feedback to the cell to cause it to make even more Rubisco. This would allow the plant to make even more sugars to survive.

Rubisco gene expression is organ-dependent

Based on our RT-PCR results of tomato leaf, stem, and root RNA we have concluded that the presence of Rubisco RNA is organ-dependent. Our results show that Rubisco RNA is most abundant in leaves. This would make sense because most photosynthesis takes place in the leaves. We have also detected Rubisco RNA in stems and roots. Stems have chlorophyll and can therefore perform photosynthesis. The result for roots is surprising. Roots typically have no exposure to light. Additionally, Rubisco requires 2 subunits – the large and the small – for this function. The gene for the small unit, which we were detecting, is stored in the nucleus. Root cells have a nucleus. However, the gene for the large Rubisco subunit is stored in the chloroplasts. So now the question is, do roots have chloroplasts? There may not be chloroplasts in root cells. If it is true maybe the small subunit has some function of its own. Or maybe the small subunit is being transported to the leaves from the roots, but this requires further investigation.

ADDENDUM

Detecting the Rubisco enzyme in bean seedlings

When we first started our project we started to test to see if the Rubisco gene was on in bean embryos. We tested many different days to see if it was actually on or not; we weren’t exactly sure whether it would even be on at all. When setting up our experiments the primers, materials and methods were made for tomato embryos but essentially could be used with bean embryos. We tested the primers on RNA isolated from mature bean leaves (see figure 11). These results showed that Rubisco could be detected in beans, but the band was not as bright as the band from tomato leaves. We then applied this process to bean embryos with mixed success. We would test several different days and it would be faintly on and when we tested it again for the same day it wouldn’t. Thinking there was an error because of the primers and materials we decided to switch over and use tomato seedlings.

References


Abstract

The goal of this experiment was to see if light had an effect on maternal aggression in LFM. Female mice are rarely aggressive, but when a lactating mother is faced with a male intruder, she will attack the male as a way of protecting her pups. This behavior is called maternal aggression. This is an evolutionary response because a male intruder will attempt to kill the pups as a way of passing down his own genes. We hypothesized that mice exposed to significantly higher light levels were going to be less aggressive. Light causes stress in mice and stress causes a decrease in maternal aggression. This experiment calls for laboratory mice to be shelved in three different light levels, high light (top), moderate light (middle), and moderate darkness (bottom). Data collected consisted of mom and pup weights, activity pre partum, maternal behaviors, maternal aggression, pup retrieval and elevated plus maze performance. The original hypothesis was not supported; light did not have a significant impact on behaviors, though location did. We determined that light level in the testing room was not important; animals tested in both light and dark conditions performed the same. The impact of location in the home room was seen with maternal aggression (mice on the bottom row were less aggressive than the top and middle rows), litter size (mice on the bottom row had larger litters), pup retrieval (moms on the bottom row retrieved faster) and change in pup weight (pups on the bottom row grew faster). We believe this change in behavior was a result of environmental variables present on the bottom row, including noise, smells, temperature and external activity level (room cleaning, cage relocation). In conclusion, we believe that all environmental variables should be taken into careful consideration before an experiment is conducted and that the bottom row should be avoided.

Key Words:

Mice, mouse, aggression, maternal aggression, light, anxiety, stress

Introduction

The intent of this research project is to examine the role of lighting as a modifier of maternal aggression in lactating female mice (LFM). This paper will serve as an analysis of an experiment conducted over the course of summer 2006. In broad terms, maternal aggression is an instinctual behavior that LFM use to protect their pups. The main purpose of this experiment is to determine if there is a relationship between light levels and maternal aggression, a correlation that has never been thoroughly investigated. This paper begins with a synopsis of our topic and some fundamental knowledge that is necessary for the understanding of stress, light and aggression in mice. Lastly, this paper is written so that it that describes our experiment in great detail, and is designed so that the study can be replicated to continue and further our research. We believe that since exposure to light has been proven to create anxiety in mice, and that anxiety has been proven to trigger a decrease in maternal aggression, then light will cause a decrease in maternal aggression.

Maternal aggression is a behavior that is exhibited in lactating female rodents. The Gammie Lab, located in the Zoology Department of the University of Wisconsin in Madison, is dedicated to studying the neural basis of maternal aggression in female laboratory mice. Aggression in non-LFM is rare; however, a LFM will often display confrontational or violent behavior towards a male intruder. This attack behavior is not true for virgin female mice because they are generally fearful of the larger male (1). The reason behind the aggression of lactating females stems from an instinct to protect her pups. Male mice are infanticidal towards pups that are not genetically related to them. This allows the male to eliminate another male’s genetic material from the population. If the male is successful in eradicating the litter, he will then be able mate with the mother and thereby pass down his own genetic material (2/3).

Previous studies suggest a direct connection between high anxiety/fear and diminished aggression in a LFM. The conclusions to these studies support the theory that a LFM subjected to stressors will be less aggressive than a LFM subjected to no stress (4). This is because of a neuropeptide referred to as the corticotropin-releasing factor (CRF) which is known to increase fear and anxiety (4). LFM become less sensitive to increasing levels of CRF, therefore causing them to be less anxious, allowing the female mouse to be more aggressive (2/4). This sensitivity to CRF occurs only when a female mouse is lactating, making the LFM much more likely to protect her pups from a male mouse that is much bigger than her.

Most people assume that higher stress levels lead to higher aggression. We believe it is the term “stress” that arouses this confusion. For example, a human who would be deemed
“stressed” is often seen as somebody who will exhibit angry and violent behavior, quite the contrary to what is found in mice. So, is the neurological system of a mouse completely opposite from that of a human? The answer is no; in fact part of the reason that scientists study mice is because of the commonalities between the two neurological systems. In truth, this misunderstanding lies within the interpretation of “stress”. When lay people say “stressed” they are referring to the physiological indicators commonly associated with high anxiety and fear, such as increased respiration, heart rate, and mental overload. In mouse studies, stress is a term used to describe the application of unusual outside stimuli, such as predator odor, light, and loud sounds; which cause the physiological changes mentioned above. Stress is often used to induce fear and anxiety in a test organism. In the animal kingdom, as is true with humans, fear and anxiety are more likely to induce a “flight” response, whereas high levels of confidence and comfort are more likely to induce a “fight” response.

Mice are nocturnal creatures, which means that the dark is a more favorable and secure environment for them. Light makes them more visible, and therefore more vulnerable to predators. Previous studies indicate that light causes stress in mice. Mice exposed to light for 24 hours causes a significant increase in anxiety and fear, as measured by their Elevated Plus Maze performance (6). A common technique for measuring anxiety and fear in the laboratory is to give mice a choice between light and dark environments after applying a stressor (6). These experiments show that anxious mice tend to spend more time in the closed (dark) part of the environment, so we infer that light induces anxiety/fear in mice.

As we have discussed, anxiety and fear triggered by stress affects maternal aggression. The evidence also suggests that light causes anxiety. We hypothesize that there will be a correlation between increased light level and decreased maternal aggression.

**Materials and Methods**

One virgin female ICR mouse and one sexually mature ICR male mouse were each placed in 45 cages. The cages are 13” X 7” and are made of polypropylene plastic, had a wired metal top equipped with slots for food and water, and contained a handful of shredded wood bedding. The animals were only placed in either a female or a neutral cage. The mice were fed Harlan Breeder Chow and given water through elevated water bottles, inserted at an angle. Each rack contained 7 cages on the front side and 8 on the back side of each shelf, 15 cages were placed on the 1st, 3rd, and bottom shelves of a 6-foot-5-shelf- metal rack (see Figure 1).

The rack was positioned under a source of fluorescent light on a 14-10 light/dark cycle. After placing the animals of the rack, the light levels were measured, in Lux, using a Traceable™ keychain light meter to determine the distribution of light among the cages. Behavior observations of the 90 mice began on the Day 4 after pairing. During these observations, the animals were visually determined to be either active or inactive. On the Day 10 after pairing, the male mice were removed and a compressed cotton pad was placed with each female. This will be used to check for activity in the pregnant females. Starting on Day 19 after pairing, maternal behavior were observed. During these observations we observed the number of times the mother nursed, groomed both the pups and herself, stayed on and off the nest, ate, and drank.

On the Day 5 and 6 postpartum, maternal aggression tests were run. On Day 5, maternal aggression tests were conducted in dim light for one half of the moms and normal light for the other half, switching the order on Day 6. In order
to conduct the test in the most efficient manner, the pups were removed from the cage via gloved human hands, while a screen shields their removal from their mother. After removing the pups, a sexually naïve male intruder was introduced into the mom’s cage. Maternal aggression attacks were scored based upon 3 principles; time to first attack, total attacks, and total time aggressive. The aggression tests were recorded for later scoring by lab personnel.

On the Day 7, the mom’s underwent elevated plus-maze tests to corroborate their stress level. A plus-maze is made of black Plexiglas and consists of two open arms (30 X 5 cm) and two closed arms of the same dimensions with 30 cm walls. It was placed 50 cm off the ground and the arms connected by a 5 X 5 cm square. The mice are placed in the center of the maze and could move at their own will.

Results

Room setup
After setting up the room, we took light readings from each individual cage. Analysis of the data showed that light levels and location were significantly correlated, and that there was a significant difference between the top row and the middle and bottom rows. There was no significant difference in light between the middle and bottom rows. This established that each row had varying light levels (top 1,051.2 ± 35.1, middle 37.6 ± 19.5, bottom 12.2 ± 15.2) (See Figure 3).

Pre-birth behaviors
Day 10 after pairing, we observed the female mice in their cages and evaluated the data to see if there was a difference in behaviors between the different locations; we found no significant correlation. (Table 1)

Birth statistics
On Day 0 postpartum, we weighed both moms and pups, and counted the number of pups. We compared location to birth after pairing (the number of days after initial pairing with male), mom’s weight, and pup weight and did not find any significant relationships. (Table 2)

Location and litter size
After collecting the data for the litter sizes of all moms, we evaluated the relationship between location and litter size and found significant correlation. The data indicates that moms on the bottom row had larger litters when compared to the top

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Top Row</th>
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<th>Bottom Row</th>
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<tbody>
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<td>% Behavior</td>
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<td>Std. Err.</td>
<td>Mean</td>
</tr>
<tr>
<td>Sleeping</td>
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<td>90.7</td>
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<tr>
<td>Self grooming</td>
<td>5.3</td>
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<td>4.2</td>
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<tr>
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<td>1.1</td>
</tr>
<tr>
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<td>0.1</td>
<td>0.3</td>
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<td>1.7</td>
</tr>
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<tbody>
<tr>
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<td>Std. Err.</td>
<td>Mean</td>
<td>Std. Err.</td>
</tr>
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<td>2.0</td>
</tr>
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</table>
and bottom rows (top, 12.6 ± 0.68, middle, 12.8 ± 0.82, bottom, 14.3 ± 0.45) (See Figure 4).

**Maternal behaviors**

On the Day 3 after birth we observed maternal behaviors. The percentage of time each mom spent doing the following was calculated: Total time on nest, off nest, nursing, high arch nursing, licking and grooming her pups, self grooming, eating and drinking, off nest doing nothing, and retrieving pups. There were no significant relationships between either light level, or location, and maternal behaviors. There was a directional difference in on nest time between the bottom and top row, with the bottom row animals spending more time on nest. Interestingly, the top row moms spent more time nursing, and directionally more time high arched back nursing but this did not seem to translate to pup weight change as will be discussed later. (Table 3)

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Top Row</th>
<th>Middle Row</th>
<th>Bottom Row</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Behavior</td>
<td>Mean</td>
<td>Std. Err.</td>
<td>Mean</td>
</tr>
<tr>
<td>On Nest</td>
<td>17.0</td>
<td>2.60</td>
<td>20.0</td>
</tr>
<tr>
<td>Off Nest</td>
<td>25.4</td>
<td>6.4</td>
<td>29.5</td>
</tr>
<tr>
<td>Total Nursing</td>
<td>58.0</td>
<td>6.0</td>
<td>52.0</td>
</tr>
<tr>
<td>High Arched Back Nursing</td>
<td>18.0</td>
<td>4.0</td>
<td>12.3</td>
</tr>
<tr>
<td>Licking and Grooming</td>
<td>10.2</td>
<td>2.0</td>
<td>12.2</td>
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<td>Self Grooming</td>
<td>05</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Eating/Drinking</td>
<td>8.0</td>
<td>2.4</td>
<td>10.6</td>
</tr>
<tr>
<td>Off Nest nothing</td>
<td>10.2</td>
<td>4.0</td>
<td>12.2</td>
</tr>
<tr>
<td>Pup Retrieval</td>
<td>01</td>
<td>0.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Location and aggression**

After scoring the aggression tests, we evaluated the data between the levels of aggression and location. Aggression was measured by time to first attack, total number of attacks and total time of attacks. We found that only value measured as significant was the total time aggressive, as discussed below. (Table 4)

<table>
<thead>
<tr>
<th>% Behavior</th>
<th>Top Row</th>
<th>Middle Row</th>
<th>Bottom Row</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to 1st Attack</td>
<td>Mean</td>
<td>Std. Err.</td>
<td>Mean</td>
</tr>
<tr>
<td>Total Attacks</td>
<td>7.0</td>
<td>1.4</td>
<td>7.5</td>
</tr>
</tbody>
</table>

After running and scoring tests, we evaluated the data between the total time aggressive and the location of the mice. This correlation came out significant. This shows that moms on the top row were more aggressive (top, 40.3 ± 4.3, middle, 39.5 ± 4.5, bottom 26.0 ± 3.8) (See Figure 5)

**Light level in testing room and total time aggressive**

We evaluated maternal aggression data to see if there was a difference when animals were tested with the lights off or on in the testing room; the results were identical and show that testing room lighting does not matter. (See Figure 6).
**Location and retrieval of 1st pup**
We measured the time it took the moms to retrieve their 1st pup in the nest after the maternal aggression tests, and ran a correlation between time of retrieval and location; this was significant. Here we can see that moms in the bottom row retrieved much faster than other moms (top, 71.9 ± 25.1, middle, 61.5 ± 29.7, bottom, 21.7 ± 16.9) (See Figure 7). We also ran a correlation between location and time of retrieval for the 4th pup; this was also significant. Here we can also see that moms in the bottom row retrieved much faster than other moms (top, 109.3 ± 23.9, middle, 91.7 ± 38.6, bottom, 56.4 ± 44.4) (See Figure 8)

**Location and elevated plus maze**
The elevated plus maze tests were scored by taking into consideration the time each mom spent in the closed arms, the middle, and open arms of the maze. Correlations were run between each EPM location and location on the rack. This data was not significant. (Table 5).

**Location and weight per pup on Day 7**
After weighing the pups on Day 7, we ran a correlation between the weights and the different locations of the litters; there was no correlation. This shows that pups from moms on the bottom row were bigger on Day 7 (top, 4.6 ± 0.6, middle, 5.2 ± 0.7, bottom, 5.3 ± 0.9) (See Figure 9)
**Location and Pup Weight change**

After calculating the difference between pup weight on Day 0 and Day 7, we ran a correlation between location and pup growth; this was significant, which means that pups from moms on the bottom row grew faster (top, 3.1 ± 0.7, middle, 2.9 ± 0.6, bottom, 4.1 ± 0.9) (See Figure 10)

![Location versus Proportion Change in Pup Weight](image)

---

**Discussion**

Our hypothesis was not supported by the data. We hypothesized that moms on the top row would have the least aggression due to the fact that they have been stressed by light, but we saw the opposite of what we expected to see; moms on the bottom were less aggressive. We were able to come to some interesting conclusions about lighting and animal room conditions. Among these conclusions were the significant relationships between location and light, litter size, total time aggressive, pup retrieval and pup growth.

We first looked at the results for location and light level (Figure 3) by averaging the individual cage light levels for each row. The middle and bottom rows were nearly identical in terms of light level; the only row which stood out as different was the top. By establishing this relationship, we were able to run statistics based upon location (top, middle or bottom) and to identify differences in behavior associated with row location. Knowing this also allowed us to assume that any difference in behavior between the middle and bottom rows was not due to lighting, but could be due to other environmental variables.

Pre-partum behaviors were observed and no significant differences were found based upon either location or light level. We believe this is due to the fact that the animals were heavily pregnant (Day 12 post pairing) and spent the majority of their time sleeping (+90%). This may be different if the animals were observed at an earlier time in their pregnancy.

At Day 0 post partum we began to see differences influenced by location. We measured litter size, mom weight and pup weight on the Day 0. For litter size (Figure 4), we saw that the bottom significantly differed from the middle and top rows, with the bottom row yielding larger litters. This was not a function of mom weight, as mom weights were all comparable between the rows. The significant difference in litter size also translated to a significant difference in pup weight on the bottom row since moms with larger litters tend to have smaller pups. It is difficult to conjecture why this litter size is different, and may just be an artifact of the small sample (n=13). Most fascinating is the concept that whatever environmental factors are contributing to the overall behavioral differences also seem to be at play prior to the moms giving birth.

Maternal behaviors were observed on Day 3 post partum. We were able to see a directional difference between top and bottom rows, and that animals on the bottom were on nest more than the animals on the top. We believe this is due to the fact that the animals on the bottom seemed more anxious than those on the top. We believe that they spent more time on nest, and as a consequence nursed their pups because they were anxious and attempting to protect them.

Aggression tests were run on Days 5 and 6 post partum. We measured the time to first attack, the total attacks, and the total attack time. We found that location did not have a significant affect on the time to first attack and the total attacks, yet it had a significant affect on the total attack time (Figure 5). Animals on the bottom row were significantly less aggressive than either of the other rows. We believe that moms might have been less aggressive because they were stressed by factors other than light, such as human feet walking through the room, bumping and jostling before and after tests due to the fact that they were on the bottom and hard to reach, different air currents, more smells settling to the bottom of the room and away from the air takeaway vents. All of these factors could quite well have contributed to the difference in anxiety level and hence, aggression.

Pup retrieval was conducted immediately following the maternal aggression tests. The results show the top row was the slowest to retrieve the pups, the middle row following, and the bottom row having the fastest retrieving moms (Figure 7). There appears to be a trend in the speed of retrieval from bottom to top row. Light may not have a direct affect on aggression, however it may be generating anxiety within the mouse is that is exhibited in the pup retrieval trials. This anxiety may cause the moms to be more protective of their pups.
The elevated plus maze test was conducted the Day 7. None of the data was significant, but it appears that animals on the bottom row were marginally more stressed, the top is the least stressed and the middle row once again lies in between them, however similar to the top row (Table 5). Animals on the top row spent directionally more time in the open and middle sections of the maze as compared to the bottom row. This data seems to support the aggression data, in that it directionally indicates anxiety in the bottom row animals.

On Day 7 the pups were weighed again. Pups on the top row were significantly smaller than those on the bottom and middle rows (Figure 9). The top row moms spent directionally less time on nest than the bottom row moms. This may also be a function of other environmental conditions in the room; perhaps the temperature was higher at the top. Maternal behaviors may also be affected more by light than maternal aggression. This is an interesting area for future research.

We also calculated the pup weight change over time for each row and compared this change to the location in the room (Figure 10). Pups on the bottom row grew significantly faster than those on the top and middle rows. This may be explained by the fact that moms on the bottom row spent directionally more time on nest than the middle and the top rows, (Table 3). As discussed previously, the increased anxiety level of moms on the bottom row may be causing them to spend more time on nest protecting their pups.

In conclusion, though our original hypothesis was not supported we did find many interesting relationships between light, environment and mouse behavior. This is the art of science, sometimes you find something, sometimes you find nothing, but rarely will you find exactly what you are looking for. In this particular experiment, although light did not have a significant effect on maternal aggression, we did discover that it affected maternal behaviors, another very important part of studying mice. Future scientists may look at the data we compiled in order to further the study of mice.

Summary

- Location had an effect on all of the following:
  - Animals on the bottom row had larger litter sizes.
  - Pups from animals on the bottom row grew faster.
  - Animals on the bottom row were significantly less aggressive.
- Light levels did not seem to matter in terms of maternal aggression or behaviors.
  - Light levels in the testing room did not have a significant effect on maternal aggression

Conclusions

- The bottom row of the racks should be avoided when housing animals for maternal behavior and aggression testing.
- Care should be taken when arranging animals for testing. The impact of all environmental variables should be evaluated prior to housing animals.

Acknowledgements

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The Effect of Bacteria on Children’s Health

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Abstracts

The goal of this research was to determine whether children get sick more frequently from spending time inside or outside. In order to investigate this question surveys were conducted which attempted to capture the health history of children under the age of eighteen. The relative amounts of bacteria from inside and outside environments were observed to determine where more bacteria exists. It was expected that children who spend more of their time outside will be sick more frequently. Similarly, it was hypothesized that bacteria will be more abundant in outdoor environments. Results did not support the hypothesis, indicating that children who spend most of their time inside get sick more frequently than those who spend their time outside, though more bacteria exists outside.

Introduction

Bacteria are the most abundant organisms that exist in the world; they are so tiny that it is impossible to see them with the bare eye. When an individual comes into contact with pathogenic bacteria there is a possibility for him/her to catch an illness. Since bacteria are all around us, we are hoping to find a location with the greatest amount of bacteria, which may be either in an inside environment such as a house or building, or an outside environment such as a park or garden. Both children and adults get sick, but we focus on how bacterial exposure has an effect on children’s health. We believe that children who are exposed to an outside environment rather than an inside environment have a higher chance of getting sick because we believe that there is more pathogenic bacteria in an outside environment. By exploring our hypothesis it will be useful to help parents become more aware of their children’s health. People will notice how much bacteria has an effect on their health. Individuals are exposed to many objects without realizing how much bacteria that item has. For example, objects that are used often such as cell phones, bathrooms doors and the handle.

The focus of our project is to explore the immunology aspect of bacteriology. Immunology is the study of the body’s natural defense mechanism against infection and disease. Microorganisms are miniature organisms such as a bacteria or a virus. The purpose of the immune system is to try to demolish any microorganism that tries to alter the body. The immune system has three lines of defense. First, “The nose, which has skin and mucous membranes to prevent any microorganisms from entering the body. Next is the innate or non-specific immunity” The innate or nonspecific immunity is when cells act on any particle in the body that is recognized as foreign. This system destroys the particle as a protection mechanism. The bodies third defense is when either the lymphocytes or the white blood cells which comes from the bone marrow or thymus arrive to destroy the microorganisms. The final defense mechanism would try to get rid specific invaders, but it takes longer to identify and destroy it. When the body is able to respond to foreign microorganisms, the immune system is working properly. Another way you can tell if it works properly is when the immune system activates before any tissue or body part has been affected. If there are no pathogens that enter the body then the immune system would not activate. Pathogens can be bacteria, fungus, or viruses that can cause disease in living organisms.

There are many different types of microorganisms that impact individuals’ health. In the 20th century, Influenza killed 50 million people (Dyer, Owen). The flu is a virus that invades the body by producing virus strains, the flu can alter itself into two-hundred different forms that the immune system is not able to recognize as identical (Davidson, Tish). It has been stated that “More than 1 in every 500 U.S. citizens are born with an immune system defect” (Starnbach, Michael N). An example when the immune system goes wrong is when a person is born with allergies. Allergies may be triggered by dust, mold, or plants. Research shows that diarrheal diseases annually kill an estimated four to six million children worldwide to the influenza that leads to 30,600 deaths each year in the United States. (Michael N. Starnbach).

We believe that there are more bacteria outside rather than inside because the information that we have gathered gives us an understanding that individuals exposed to different environments have a higher variety of contracting different
types of microorganisms. For our study, we distinguish pathogenic from non-pathogenic bacteria but assume that the relative amounts would be similar in the two environments. It has been proven that individuals who live at a residence where there is a lot of dust, mold and old materials have a greater amount of exposure to pathogens. (Dyer, et al). Cold weather and muggy places is an ideal for bacteria to grow since it has essential conditions for the bacteria to grow. In addition, “places that are damp, moldy and overcrowded can lead to greater rates of infection, allergies, and injuries, respiratory and skin disease” (Kuhn D). Indoor mold can cause flu or the common cold because mold is bacterium that people breathe in. People tend to get cold when their family members do because of the times they spend together under one roof. Not only do people catch colds from their surroundings but the most common way to catch a cold is by handshaking as in hand to hand contact. When a child has an illness that the heat will be trapped inside the body and the body will start to have a fever.

If the parents are healthy, the children have a good chance of being born healthy, because the mother can pass all of her antigens to her child by breast feeding the infant. Eventually, the child will get sick when they are exposed to bacteria that the mother never encountered, and therefore does not have antigens for. Even if children are born healthy, they still need vitamins in order to have a better health. Children like to touch everything they see, this causes them to be at greater risk for catching an illness. Adults are less likely to catch the illness because they were already exposed to those pathogens when they were younger. Therefore, the minors are more likely to become ill because they have not been exposed to the pathogens yet. Overall, children who are exposed to more pathogens when they are young are more likely to have a healthier adult life.

Methods

In order to test our hypothesis we decided to conduct a survey and perform a lab. In the survey, we came up with some questions that included the number of times sick, season of illness, as well as if it was a bacterial or viral illness. We also asked if the children spend most of their time inside or outside, and which common objects children come in contact with. These questions from the survey will help us determine where and what to swab when we perform our lab.

We decided to go to various locations in search of parents with children of 18 and under, such as Memorial Union, and State Street. We wanted to survey as many parents as possible to have the most accurate data. We asked 100 parents to take the survey, with a total of 188 children.

In order to create this lab certain factors must be met. To start we had to analyze the data that we got from the surveys in order to know where to swab for bacteria. Some objects that parents said their children came into contact with were Lego’s, playhouses, basketballs, bikes, monkey bars, earphones, etc. We tested eleven objects from inside but only seven of them had formed some bacteria and four of them didn’t show any bacteria formed. The things from the inside objects were swabbed were bathroom flush handle, legos, stuffed animal, towel, earphone, case corner clothing room, mat, dinosaur toy, soap dispenser handling, marker, and the sink handle.

Once we reviewed the objects with which children came into contact we decided to go a Madison, WI Day Care Center on July 20, 2006. We used sterile swabs to swab the objects that the parents said their children came into contact with. Twelve objects were taken from outside and twelve from the inside. The sterile swabs were opened carefully and used right away making sure the entire swab touched the designated object. Then the swab was placed back in the same package so other bacteria would stay isolated from the used swab. Every sterile swab package was labeled with the place where we used it so we would be able to identify where it came from and the name of the object that we swabbed. When we got all the bacterium that we wanted to collect we headed back to the biochemistry room to prepare the bacteria to reproduce.

To prepare the bacteria we needed the following items: Petri dish, agar, container, incubator, pen, bunsen burner and copper wire. First we heated the agar in order to melt so we could place it on the Petri dish. When the agar was melted, we position the agar inside the Petri dish. Next we let the agar cool so we would be able to swab the bacteria inside the Petri dish. We labeled the Petri dish with information on where the bacteria was taken. Once the Petri dish was labeled it was time to use the sterile swabs that contained the bacteria and swab them inside the Petri dish in a process call streaking for isolation. In order to do this we had to swab three parts of the Petri dish(See figure A1). The first part we used the bacteria swab in an upward and downward movement until it reached half of the Petri dish. Then we got a sterile swab, and swabbed the bacteria a second time, touching the old bacteria horizontally, but only touching it twice as it goes around half of the remaining portion of the Petri dish. For the remaining one third of the Petri dish we used a new sterile swab and swab it diagonally touching only the previous bacteria and not the original bacteria. By using the streaking for isolation process we were able to isolate the Petri dish from enormous amounts of bacteria, to smaller amounts every time until eventually if everything is accurate you will be able to count the CFU (Colony Forming Units). In order to count the CFU (Colony forming Units) you must make two parallel lines going up and two parallel lines going horizontally. Next you count all the bacteria that grew in every space and total the whole bacteria up. (See figure A2). By being able to count the CFU’s we will be able to find the amounts of bacteria that each environment contains.
Before the bacterium was ready to be incubated we decided to do a second sample for all the bacteria. If we have enormous amounts of bacteria it will be easier to count the CFU’s if we have a smaller amount of each bacteria. To do this we followed the same steps by preparing the Petri dish with the agar and labeling it with the same information, except we added the number two at the bottom to distinguish it from the original Petri dish. Once the Petri dishes were ready we installed the Bunsen Burner into a gas line to get a flame that would disinfect the inoculating loop. Next we placed the copper wire into the bunsen burner in order to heat it up. Once the inoculating loop was heated we took it off the bunsen burner and let it cool down in order so the bacteria would not die. When the inoculating loop was cool we opened the first Petri dish and swabbed the inoculating loop on the last third that we had swabbed, in order to get only small amount of bacteria into the second Petri dish. When we got the bacteria on the inoculating loop we swabbed it on the second Petri dish in a vertical line all around the sample. After this we repeated the same procedures for the remaining Petri dishes but kept burning the inoculating loop so our Petri dishes would not become contaminated with different types of bacteria. Finally when all the Petri dishes where done we placed them into a container to incubate. In order to incubate them we had to find the right temperature, for the bacteria to be able to duplicate which is 37 degrees Celsius. We found a room with a temperature of 37 degrees Celsius, and left the container inside. One day later, July 21, 2006 we took the container out of the incubator in order to view the bacteria that had grown.

The samples that did not grow were not placed in the chart because no CFU’S where visible. No growth was seen so we concluded that the objects were disinfected everyday or that when we swabbed an object we didn’t swab it well enough. When we were done making our observations, which took about an hour, we placed the Petri dish back into the incubator so we could see how the amount of bacteria developed. On July 24, 2006 we took the Petri dishes out of the incubator to take notes on the amounts of bacteria. When we finished we threw the Petri dish away since we had enough data about the amount of bacteria in each environment.

Analysis

The data that was provided from the survey was examined and was it is shown in chart (A1). In order to view this data three factors must be understood, The first factor is the number of times children get sick. By looking at the chart one can determine that the fewest times a child got sick was seven times. In addition, one can formulate that children generally get sick between one and two times a year. The second factor is the environment in which the children were. The final factor is the amount of children which is 183. By looking at the graph we can easily compare that more children that spend their time inside the environment instead of outside. We calculated the total percent of individuals who spent their time inside and outside. First we added all the ins and outs and then divided by the total which then we multiply in order to get our percentage. Our analysis shows that 28.4% of children spend their time outside and 65.6% of the children spend their time inside. Finally children that spend equal amounts of time in both environments were 6%. 28% of the children that when outside have a greater chance of getting sick but not as many as the children who spend most of their time inside. Because our chart indicates 65% of the children spend more of their time inside, it can be concluded that they get sick most often.
As for the season of illness, there is a big difference in winter because it has the highest percent of children who had the illness (58.58%) but during the summer, there was a much larger percentage of sickness that occurred. Spring and fall had about the same percentage of sickness. There were 18.6% of those who were sick during fall and about 15.1% were sick in spring. Viral infections caused more sickness in winter than in summer. Now, we know that children under the age of 18 have a lower chance of getting sick from bacterial infections during summer rather than the winter. Additionally, children will most likely have an equal amount of sickness during spring and fall, but they have greater chance of catching viral illnesses in winter. In the spring, there isn’t much of a big difference between the bacterial or viral. Of all the comparisons between the bars of the bacterial and the viral that effected in a season shown in the graph that it tells us that children will be most likely to get sick by the cause of viral rather than bacterial illness.

By looking at figure (B3) we can examine the amount of time that males and females spend in an environment. The results show that most children spend their time inside the environment rather than outside. A greater number of females spend more time inside rather than outside. 36% of the females spend their time inside, males only spend 17% inside. Males spend more time outside. For example, 32% of the males spend their time outside while females spend approximately 14% of their time outside. With information from the graph, we can say that girls spend most of their time inside rather than an outside environment. However, there isn’t that much of a difference between the amount of females and males who spend their time inside and outside.

From the inside objects, earphones had the most colonies of bacteria indicated and the least bacteria shown was from the different shapes such as round, and snow flake shaped appearance. The bacteria from the bathroom flush handle had only two round shaped bacteria attached together in the first streak. The dinosaur toy, soap dispenser handle, marker, and sink handle didn’t show any growth. We believe there were some errors made on the objects that didn’t show any bacteria because if the sterile stick was swabbed on the object only on one side and not all around the cotton head. That would have been the problem if the sterile stick was swabbed on the plate from the side that was swabbed from the item. Overall the results of our experiment indicate that objects from the outside have a greater amount of bacteria than objects from the inside. Objects outside are usually sterilized which may explain some of this difference.
Results

After leaving all twenty-three swabbed nutrient agar plates in the incubator for eighteen hours, no bacteria had formed. There were eight plates that showed some growth of bacteria. Eight of them were from outside and four of them were from the inside environment. From the observation of the plates, the objects that had most growth in the inside environment were the earphones. The greatest amount of growth from the outside environment was the playhouse. (See A3). The bacteria from the earphones had about 20 tiny colonies of bacteria, and the sandbox car had 25 colonies of bacteria shown. We figured out that eighteen hours of incubation was not enough time for the bacteria formation so we put all of the samples back in the incubator for the weekend. After incubating for another sixty-six hours, it showed some changes on both the inside and outside dishes that were swabbed. Even though bacteria formed in some of the plates, some other plates did not show any bacteria. There was a great amount of difference in the amount of bacteria in the dishes than sixty-six hours earlier. We used the process of streaking for isolation in order to observe the differences between the amounts of growth of bacteria. Plates that were swabbed from inside objects showed fewer bacteria than the outside objects, which showed more bacteria.

The objects that we swabbed from the outside were slide on the side handle, playhouse window, monkey bars, table, sandbox bar, basketball, steering wheel, bike seat, hula-hoop, swing handle chain, and wheel barrel truck. Of these objects results show that the most bacteria came from the playhouse window and the least bacteria came from the hula-hoop. From all the above objects that were tested, the playhouse window had a lot of bacteria all around the plate. Some of the bacteria had sphere shaped in the first streak and a big chunk of bacteria in the second streak with an orange dot of bacteria, yet there were some spread out bacteria in the third streak. The bacteria from the hula-hoop had some tiny pink dots in the first streak but in the second streak, there was only one tiny CFU. All together, there were about eight colonies of bacteria and one of them was in the second part of the streak. There weren’t any bacteria formed in the third streak which might be a reason because there weren’t enough bacteria caught on the sterile stick when the object was swabbed.

Discussion

The information from our survey indicated children were sick more were ones that spent most of their time inside. The survey shows children that have allergies get sick more often than those who don’t have allergies. Children who spend most their time inside were the ones with more frequent sickness and allergies than those who spend their most time outside.

<table>
<thead>
<tr>
<th>Object</th>
<th>Inside or outside</th>
<th>Colony Forming Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Legos</td>
<td>In</td>
<td>2</td>
</tr>
<tr>
<td>Play house window</td>
<td>Out</td>
<td>Enormous amounts visible strings</td>
</tr>
<tr>
<td>Swing handle chain</td>
<td>Out</td>
<td>5 scatter units</td>
</tr>
<tr>
<td>Container case</td>
<td>In</td>
<td>3</td>
</tr>
<tr>
<td>Wheel steering</td>
<td>Out</td>
<td>2 enormous colonies</td>
</tr>
<tr>
<td>Basketball</td>
<td>Out</td>
<td>1 colony</td>
</tr>
<tr>
<td>Bike seat</td>
<td>Out</td>
<td>3</td>
</tr>
<tr>
<td>Sand box car</td>
<td>Out</td>
<td>25 made in a curb</td>
</tr>
<tr>
<td>Wheel barrow truck</td>
<td>Out</td>
<td>14 scatter around</td>
</tr>
<tr>
<td>Monkey bars</td>
<td>Out</td>
<td>Enormous amount on top</td>
</tr>
<tr>
<td>Towel</td>
<td>In</td>
<td>3 top middle</td>
</tr>
<tr>
<td>Earphones</td>
<td>In</td>
<td>20 CFU scatter around</td>
</tr>
</tbody>
</table>

We believe there were more bacteria on the outside objects because outside environments are contaminated and that people inside objects since it is a closed environment. Overall, we can conclude our hypothesis from the surveys and the lab that objects from the outside environment come out to have more bacteria compared to the inside environment. We hypothesized that children who spend more of their time outside have a greater chance of getting sick. However, our survey shows that there were more of children who spend most time inside get sick more often than those who spend most of their time outside. This may indicate that there’s more pathogenic bacteria living inside rather than outside. Even if some of the swabbed plates didn’t show any result, we can still proceed with our conclusion from those plates that did show some changes. We can examine our results not only from the lab but from the surveys that were completed.

The surveys and tests indicate that children who spend most of their time inside have greater chance of getting sick than those who spend most of their time outside. From looking through all of the surveys that were answered, there was no difference between the age and the amount of sickness.

When we moved on to our experiment we saw extraordinary results. For example, in the lab we didn’t see much growth of bacteria in the plates from the objects that we had swabbed since bacteria are everywhere. Furthermore, we were able to
compare the amount of bacteria from one environment to another in our results. In our experiment only half of the 23 dishes that we prepared grew bacteria. We believe that the other eleven plates had no growth because we didn’t swab the entire cotton head of the sterile swab into the object. This meant that when we swab the bacteria into the Petri dish we had probably wiped the clean part of the sterile swab and not the part that had bacteria onto the plates. Another factor which might have affected the amount of bacteria that we collected could be the climate in the outside environment. When we collected our bacteria it was a rainy day which meant that our amounts of bacteria could have been affected because the rain might have had an impact on different types of bacteria which might have never been present on a sunny day. Similarly, when it is raining there might be bacteria present which were not present when children are on the playground. If future groups would start on our data they could use the amounts of bacteria that we collected in the daycare and the formation of the bacteria from one day to another. We kept track of the amount of bacteria that grew day by day. This information might help individuals to investigate how bacteria grow in different environments. Another idea, that future groups could investigate, is the type of bacteria that grows in the Petri dish and check if it has any impact on the children’s health. In conclusion, we were able to answer our hypothesis, but we could have swabbed more objects in order to get more accurate representation.

For further studies, another idea which could be investigated is about parent’s health history, in order to see if their health has an impact on their children’s health. Future groups might want to explore different questions in order to investigate the health of parents in order to determine if there is a correlation. A long term investigation could include keeping track of the same children’s health background to furthermore investigate whether they get sick less often as they age.

**Conclusion**

With all the experiments that we have completed, we have come up with a conclusion by analyzing the survey, labs, and data which we collected. What we have hypothesized did not match our conclusion, but the information that we did obtain allowed us to draw conclusions. We found greater amounts of bacteria on outside objects compared to inside objects. These findings support part of our hypothesis, which stated there would be a greater amount of bacteria outside. However those who spend most of their time outside have a better chance of getting sick than those who spend most of their time inside. Surveys found that children who spend more time inside get sick more often then children who spend more time outside. The information also indicated that fewer children who spend most of their time outside and those children got sick less often. Our results show that there is a greater amount of bacteria outside than the inside. However children get sick more often when they spend most of their time inside.

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The Effects of the Neuropeptide DGYRPLQFa on the muscle tissue of Ascaris suum

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Mentor: John McKenna
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Abstract

Previous studies have shown that locomotion is a good target for antiparasitic drugs. When the neuropeptide DGYRPLQFa was injected into living A. suum it caused a ventral coiling of the nematode. The neuropeptide was applied to dorsal and ventral muscle strips of A. suum and the responses were recorded. It was found that there was no statistical difference in the maximum contraction between the ventral and dorsal muscle tissues. Future studies should look at the issue of commissures which may play a role in the ventral coil and therefore help discover a better and safer method to treat Ascaris infection.

Keywords

Ascaris suum, Parasite, DGYRPLQFa, Neuropeptide, Neurotransmitter, Acetylcholine

Introduction

Parasites are known to infect large populations around the globe. Four and a half billion people worldwide are infected with some sort of parasite. Treatments for these infections are not abundant. However, parasites like other pathogens exhibit the ability to gain resistance to some drugs not only in humans, but in livestock as well. Ascaris suum is a parasitic nematode (round worm) that infects pigs. Its sister species, Ascaris lumbricoides, infects 20% of the world’s human population, and is believed to be almost identical to A. suum. The study of A. suum is very important because it may lead to the development of better safer anthelmintics, drugs which kill the parasitic nematodes. Therefore, this study is also important believe it may lead to a better understanding of a simple nervous system.

A. suum is a great model organism for studying the nervous system, because it is composed of about 300 neurons and these neurons contain very few branch points, and most of them have no branch points at all. The Human nervous system on the other hand has billions of neurons, each with thousands of possible branching points (Walrond et. al., 1985).

A. suum also has many similarities to other nematodes. This is very useful because the research acquired in this field can be applied to other parasitic nematodes in the future and the new drugs may work across species lines. Also, A. suum’s size makes it excellent for the level analysis that will be involved in this research. An adult female is anywhere from 12-18 inches long. In essence, A. suum is the most reliable asset in the research of the nervous system of parasitic nematodes (Walrond et. al., 1985).

There are two distinct muscles separated by lateral lines, the ventral (bottom) and dorsal (top) on the nematode (refer to figure1). These are innervated by motorneuron processes in the nerve cords. There are five neuronal types in the motor nervous system: four are motorneurons and only one is interneurons. The Motor neuron types are: DE, DI, VE and VI. The class called DI is inhibitory to the dorsal muscle cells. The class called DE is excitatory to the dorsal muscle cells. The class called VI is inhibitory to the ventral muscle cells. The class called VE is excitatory to the ventral muscle cells. The interneurons connect one neuron to another.

Unlike many other animals, the muscle tissue in Ascaris sends muscle arms which then form synapses with the motorneuron processes. The ventral and dorsal nerve cords are linked together by commissures. They are arranged in reproducible patterns in A. suum. Through the use of excitatory and inhibitory neuronal signals, the dorsal and ventral muscles work together to create locomotion (Stretton et. al., 1991). Shown in the diagram below is an example of a commissure leaving the ventral nerve cord and traveling to the dorsal nerve cord.
Neurons have chemical synapses which allow one neuron to send a signal to another via neurotransmitters. A neurotransmitter is a chemical released into a synapse and received by a receptor, thereby allowing a signal to be sent from one neuron to another or from a neuron to a muscle cell (refer to figure 2). Within the nervous system are various receptors for receiving neurotransmitters, which fall under two specific categories: metabotropic receptors, and ionotropic receptors. Both of these have the same function of opening ion channels, but they differ both in speed and range of effect. Metabotropic receptors, though slower in opening up a channel, have a more wide-spread and longer lasting effect than ionotropic receptors and work via a cascade of effects. Ionotropic receptors are much faster in opening a channel because they are directly connected to the channel, but also have a much smaller area and duration of effect, limited to only the immediate region of the receptor, and lasting only a few milliseconds.

**Figure 1.** The connection between dorsal/ventral and excitatory/inhibitory neurons. A filled-in triangle represents an excitatory synapse, and an empty triangle represents an inhibitory synapse (Stretton et al. 1992).

Neuropeptides are a type of neurotransmitter, which are excretions of amino acids from axon terminal buttons. These neuropeptide can cause many different effects which may be long term. Previous studies in our lab have found a many neuropeptides in the nervous tissue of *A. suum*. Current estimates have indicated that 50 – 75% of the nervous system is affected by neuropeptides in some way. The neuropeptide, which we are studying is called DGYRPLQFa and was found to be in 6 different nematode species, hinting towards a possible important function. DGYRPLQFa stands for the string of amino acids that are put together to give the peptide. Each letter symbolizes one of the twenty amino acids in the sequence (Maule et. al., 2002). Previous work done in our lab has shown that when DGYRPLQFa was injected in a live nematode, it caused a ventral coil of the body. Another chemical, Acetylcholine (ACh), causes the muscles of the *A. suum* to contract, allowing it to be used as a control to verify that muscle tissue is contracting properly.

**Figure 2.** This is an example of a commissure leaving the ventral nerve cord of *A. suum*. M symbolizes a muscle which is sending a muscle arm projection to the nerve cord. Comm. means commissure, IN means interneuron and MN means motor neuron.

The two main neurotransmitters that are used in anthelmintics to affect the motor nervous system of *A. suum* are acetylcholine and GABA. We fear that the nematodes will become resistant to these drugs, so we are researching new drugs. We feel that by studying DGYRPLQFa, we will be able to target the locomotory system of the nematode and possibly develop a better safer drug to treat Ascariis infection. This will also lead to a greater understanding of a simple nervous system.

**Methods and Materials**

Eleven trials were run by applying DGYRPLQFa to *A. suum* muscle strips. Six Dorsal Muscle Strip (DMS) trials and five ventral muscle strip (VMS) trials were conducted. First the *A. suum* nematodes were obtained at a pig slaughterhouse and kept at 37 degree Celsius during transport. Females were identified by their large size and absence of a curl in the tail. Six anterior (from the head side) centimeters were cut and pinned down. This portion was placed into a petri dish containing ringer solution, a mixture of 8mL of NaCl, 49mL of KCl, 11.8mL of CaCl$_2$, 9.8mL MgCl, 34.02g of NaC$_2$H$_3$O$_2$, and 2.093g of MOPS that mimics the internal environment of the parasite. Then either a dorsal or ventral muscle strip was dissected away. The nematode was cut down the middle of the side opposite to the desired one (i.e. ventral side was cut if dorsal muscle strip was being tested). Then each side was pinned down and the intestines were pulled out.
The lateral lines were then cut under the microscope and this separated the desired muscle strip. Silk thread was attached to each end of the muscle strip and loops were made in the thread. Then the muscle strip was placed in a vial of 37 degree Celsius ringer solution, the body temperature of the host either pig or human. One thread was attached to a hook at the bottom of the vial, and the other to a transducer above it. The transducer converted contraction or relaxation of the muscle strip into electrical signals, which were to a computer program and charted out.

Before application of the neuropeptide was attempted, a known excitatory neuromuscular transmitter (acetylcholine) was applied to the muscle strip as a control to ensure the muscle strip was functioning properly. Using a micro-pipette, 7 uL of Ach (acetylcholine) was measured and added to the test tube containing 7 mL of ringer solution making 10 uM concentration of ACh. A twenty second wait occurred and then double the volume was applied until 56 uL was applied (i.e. 14 uL, 28 uL, and finally 56 uL). During the 20 second wait the muscle strip contracted to verify that it was working properly.

After the preliminary control procedure, 7 uL of the neuropeptide was added (for a concentration of 7 uM) and the response was observed for thirty minutes. After each application of the neurotransmitters, the muscle strip was rinsed with ringer solution. Upon completion of the neuropeptide tests, ACh was applied again to verify a contractile response. Notes were recorded and results analyzed.

**Results**

Table 1 shows the maximum contraction measured for each muscle strip. The table shows the maximum contraction for the initial Acetylcholine dose response curve, the neuropeptide and final ACh dose response curve. Each number represents the maximum contraction or relaxation of the muscle strips, with either a positive or negative number respectively. All numbers were positive and therefore were maximum contractions. Each muscle strip reacted slightly differently to the application of the neurotransmitters. Therefore, to normalize the data the maximum contraction due to the neuropeptide was divided by the maximum contraction due to the first ACh dose response curve.

Table 2, “Neuro/1st ACh,” shows the average ratio for both the ventral and dorsal muscle strips. The average ratio of contraction for the ventral muscle strips was 0.5874 +/- .0554 grams of force, while the average ratio of contraction for the dorsal muscle strips was 0.4743 +/- .0709 grams of force. There is no statistical difference between these two numbers and therefore there was no statistical difference between the ratio of maximum contraction of the two muscle strips.

<table>
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<tr>
<th></th>
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<th>VMS 7/14</th>
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<th>VMS 7/20</th>
<th>DMS 7/12</th>
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<td>0.667</td>
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<table>
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<th>Ave. ratio for VMS</th>
<th>Ave. ratio for DMS</th>
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</thead>
<tbody>
<tr>
<td>0.5874 +/- .0554</td>
<td>0.4743 +/- .0709</td>
</tr>
</tbody>
</table>

Table 1. Results of all eleven trials. Each number is in grams of force and represents the maximum contraction of each muscle strip. “ACh” = Acetylcholine. “Neuro” = Neuropeptide. DGYRPLQFa. “Neuro/1st ACh” = Neuropeptide divided by Acetylcholine. “VMS” = Ventral Muscle Strip. “DMS” = Dorsal Muscle Strip.
During each trial a dose response curve was conducted using ACh. The dose response curve of ACh was very important to ensure the muscle strips were not damaged and were responding properly. The ideal ACh dose response curve showed an increased contraction when the concentration applied to the muscle strip was increased. However, some graphs showed a decrease or no change when after a large initial contraction. This was still an acceptable response as long as the muscle strip contracted noticeably (See figures 3 & 4). Figure 3 is an example of an ideal dose response curve when ACh was applied. Figure 4 shows an unexpected but still acceptable dose response curve.

Each dose response curve consisted of 10uM of ACh applied to the muscle strip. Then the dose increments were doubled until they reached 80uM of ACh (see figures 3 & 4). After rinsing, 1uM of the neuropeptide, DGYRPLQFa was applied to the muscle strip. Both the ventral and dorsal muscle strips contracted. These contractions were slow and steady showing at times slight fluctuations over the thirty minute waiting period (see figures 5 & 6). Figure 5 illustrates an expected response with a smooth contraction when 1uM DGYRPLQFa was applied to the muscle strip. Figure 6 shows some unexpected fluctuations in the response of the neuropeptide. This shows how the muscle strip contracted and then relaxed repeatedly while the neuropeptide was applied.

![Figure 3](image)

Figure 3. This is an example an expected dose response curve. Contractions during applications of ACh are shown as the cursor line moves upwards. Each vertical line through the page represents the addition of more ACh.

![Figure 4](image)

Figure 4. This is an example of an acceptable ACh dose response curve, where there is no step up in the contractions, but instead the largest contraction occurs first.

![Figure 5](image)

Figure 5. This is an example of a neuropeptide dose response curve. Both the Ventral and Dorsal Muscle strips contracted when the neuropeptide DGYRPLQFa was applied to them.

![Figure 6](image)

Figure 6. Several responses to the neuropeptide addition had the unexpected result of fluctuations in the contraction.

The final dose response curve of ACh used the same concentrations as the first dose response curve and exhibited an increases in contraction compared to the first dose response curve. Figure 7 shows a complete trial with both dose response curves and the neuropeptide response. In all eleven trials the second ACh maximum contraction was greater than the first.
Discussion

The results did not support the hypothesis that the ventral muscle would contract more than the dorsal muscle strip to explain the ventral coiling seen in previous experiments. Because there was no statistical difference between the maximum contractions of the muscle strips.

The rates of contraction of the two neurotransmitters, ACh and the neuropeptide DGYRPLQFa were different. The ACh caused an immediate contraction, while the neuropeptide caused a much slower contraction. This was expected and can be explained by the fact that the ACh has ionotropic receptors and the neuropeptide has metabotropic receptors.

In each trial the first ACh application acted as a control, because the expected contraction could be compared for each trial. Also in every trial the maximum contraction of second ACh dose response curve was larger. Therefore, the neuropeptide probably had some effect how the ACh interacted with the muscle strip. The neuropeptide might somehow have amplified the response of the ACh, even though the neuropeptide was rinsed out of the vial. This could be a source of future studies.

The results lead us to question why the ventral coil did occur if the contraction of each muscle strip while separated could not explain it. It is possible that the commissure pairs which were cut (the pass through the lateral lines and these were cut in order to separate the two muscle strips) play a role in this ventral coiling. This could be a source of other future studies.

Conclusion

Our experiments and data did not support our hypothesis, but they did broaden some of the general knowledge of the nervous system of *A. suum*. The experiments and trials also eliminated several possibilities for the ventral coil. The more that is learned about the nervous and locomotory system of *A. suum*, the better chance we will have to make improved anthelmintics and further understand complex nervous systems such as the extremely complex human nervous system.

References


The Influence of Land use on Water Quality of Artificial Ponds in South Central Wisconsin, USA.

Students: Niema Mohammed, and Victor Adalid
Mentor: Christine Donahoe
Faculty Advisor: Stanley Dodson

Abstract

Artificial ponds are man-made ponds created to retain run-off drainage water. Not much research has been done on these ponds. In this experiment we investigated how the use of the surrounding land influences the level of nutrients, pH, and the specific conductance in the ponds, as these are all measures of water quality. We hypothesized that ponds surrounded mostly by lawns and farms would have a higher level of nutrients than those near impervious surfaces. A sample was collected from 20 artificial ponds in South central Wisconsin, filtered in the lab, and tested for Soluble Reactive Phosphorus, Nitrate, Ammonium, pH, and specific conductance. We found no significant correlations between the nutrient levels and land use. However, we did find some significant correlations between specific conductance, pH, and land use. Although our fundamental hypothesis concerning nutrients was not supported, the pH and specific conductance data indicates that the land use can affect water quality.

Introduction

Farmers usually use fertilizer to enhance natural nutrients that are already present in the soil. When precipitation occurs, such as storm water or rain run off, excess fertilizer is released into artificial ponds (“Plant Nutrient Requirements”). This can lead to eutrophication. Eutrophication is the over abundance of nutrients in the pond, causing the phytoplankton to rapidly increase. When this happens, the pond begins to have a greenish look and to produce an awful smell.

The two main nutrients that cause eutrophication are phosphorus and nitrogen. Phosphorus, the least abundant nutrient, is the most important nutrient in a plant’s life, which causes the plant to grow. The second most important nutrient for plant and algae growth is nitrogen. Plants need Nitrogen or otherwise productivity would not occur. (“Atmospheric Nitrogen as Plant Food” 328-330). Measuring the pH level will help determine how acidic or basic the pond is. The pH has to be within six to nine because it is healthier for the organism to live in (“Why ph is Important”). The use of the entire watershed affects the rate of plant growth, meaning that influence such as, fertilizer contribute towards eutrophication (“Mid-Atlantic Integrated Assessment”).

Plant activity depends on the right amount of nutrients to grow. Not enough or an over abundance of nutrients such as, nitrogen and phosphorus can have an effect on productivity which is influenced by the land use. Nitrogen contributes towards algae growth, which creates a green overlay that is viewed throughout ponds. Where as phosphorus affects rapid plant growth while encouraging blooming and root growth (“Plant Nutrients”). The problem of too much productivity arises when outside factors, such as fertilizer influence the pond’s aesthetic (Dickman 660-666).

Items such as fertilizer surround areas where artificial ponds are located. Since fertilizer consists of nutrients such as nitrogen, phosphorus, potassium, calcium, magnesium, and sulfur, it helps towards the growth of plants (“Plant Nutrient Requirements”).

Artificial ponds are those that are manmade. Their main purpose is to hold drainage water. The excess water drains from the watershed, which is all land that surrounds the pond. Many farmers use this technique to save their crops. If excess water floods the crops, plants would not survive. So, when water runs into an artificial pond it carries fertilizer, soil, and all other minerals that are included throughout the watershed (Renwick).

Plants use Soluble Reactive Phosphorus, (SRP) which is only 10% of the total phosphorus. This form of phosphorus consists of mostly inorganic orthophosphate (PO$_4$) (Shapiro 298-300). Orthophosphate is the form taken by algae. Since orthophosphate or SRP is really strong, plants or algae only need a small amount of SRP. The amount that the plants
should get has to be no more than (<5ug/L) of phosphorus (http://dipin.kent.edu/Phosphorus.htm).

The level of nitrogen can depend on local land use. “Nitrogen may come from fertilizer and animal wastes on agricultural lands, human waste from sewage treatment plants or septic systems, and lawn fertilizers used” near the pond. (Nitrogen-Understanding Lake Data). Plants can also get Nitrogen from the soil, air, or various forms of nitrogen existing in the water (Atmospheric Nitrogen as Plant Food 328-330). It can exist as nitrate (NO$_3^-$) nitrite (NO$_2^-$), and ammonium (NH$_4^+$) (“Nitrogen- Understanding Lake Data”). Since nitrogen is the fundamental factor of all organic matter. All inorganic forms of nitrogen are taken up by marine plants and algae (“Nitrogen- Understanding Lake Data”). Phosphorus and Nitrogen are not the only nutrients that are in water. One way that we could test how much minerals and nutrients are in water is by performing a test called ‘Specific Conductance’. This test determines how well the water conducts electricity at a certain temperature. Electrical conductivity is a measure of “water’s ionic activity and content” (“Specific Conductivity”). The more ionic dissolved constituents, the better water can conduct electricity. Specific Conductance not only tests for conductivity, but how temperature affects the conductivity of the water. In other words, conductivity of the same water can change depending on the temperature (“Specific Conductivity”). Water that does not contain many minerals and nutrients will have a harder time to conduct electricity. There is not a specific level where water should be. Some ponds require more nutrients and minerals, and others require less. This test can give us an idea on how contaminated the water is (Murphy). Meaning, the more minerals such as nutrients, soil, and salt that is present in the water, the more electricity is able to be conducted.

We hypothesize that in agricultural areas and lawns, nutrients levels of artificial ponds would be higher than those located in urban areas. The reason being is because of the massive land of crops, desire more fertilizer. The more farm and lawns there are, the more amount of fertilizer will be added. As far as artificial ponds near impervious surfaces, (areas where water is not able to seep through) roads, parking lots; they are not in need of fertilizer.

Nutrients play a very important part in a plant’s life. Too much can cause a plant to die, or cause eutrophication. Land use might have an influence on this effect. Understanding these relationships quiled our investigations.

**Materials and Methods**

20 water samples from artificial ponds around the Madison area were collected between the hours of 9:00am and 4:00pm in the summer of 2006. The ponds were chosen by Stanley Dodson.

In order to obtain the water samples properly, 1000ml bottles, as well as the caps, were rinsed in the pond three times before collecting the final samples. To prevent air bubbles, the water samples were collected by vertically submerging the bottle in the pond. Then, capping the bottle closed while still being submerged prevents atmospheric air to be collected, possibly altering test results. From there, the water samples were stored under ice until the tests were performed. Each sample was filtered the same day of the collection using a combination of two different filters in a lab. The two filters consisted of two different sizes which were; .7micron and 1micron. This showed how much chlorophyll and total suspended solids were in the water samples. After that was done, the pH of the water was tested by simply pouring a small amount of the pond water into a container the same day of the collection. From there, the filtered water samples were bottled and stored in the freezer until the tests were performed later on during the following weeks. The tests performed included soluble reactive phosphorus (SRP), nitrate (NO$_3^-$), and ammonium (NH$_4^+$). To do these tests, the lab protocol was followed. Next we tested for specific conductance. The way this test is done, is by placing two electrodes, with opposite charges, in the water, followed with an electrical current being sent into the water.

**Results**

When analyzing the correlation matrix we found that the nutrients we tested were not significantly affected by the land use. The correlations between NO$_3^-$, NH$_4^+$, SRP, and the percent of land used for corn, woods, soy beans, meadows, lawns, and impervious surfaces show a value lower than the critical value of .433. This indicates that the correlation between the land use and the nutrient levels was insignificant. We did find some strong correlations between specific conductance and algae. Also, pH and corn field had a value higher than the critical value. For water to be healthy for the organism in the pond, it has to be as close to six through nine on the pH scale as possible. (According to our data the healthiest pond was number 16 because of the pH reading of 7.41. The most basic pond was pond eight; because of a pH reading of ten.

Therefore, our results did not support our hypothesis. We found no correlation between the land use and the quality of the water. As you can see from the appendix the numbers that are in red means that they are correlated with each other.

**Discussion**

We found no statiscally significant relationships of land use affects on the amount of nutrients within artificial ponds.
Looking at nutrients such as nitrate, ammonium, soulable reactive phosphorus are just a few ways scientists strive to answer the problem of eutrophication. This does not mean that land use does not affect water quality, what this means is that by the way that we measured water quality there was no correlation. There are many other ways to test for water quality; we just chose to test for the nutrient levels that we tested. For example, Professor Stanley Dodson of Univ. of Wisconsin-Madison discovered that the correlation between lawns and total phosphorous was significant. This could be because when SRP gets into the water, the macrophytes immediately use up most of the SRP. Therefore further studies are required to see if land use has an affect on the quality of the water.

Precipitation patterns could have affected our results. When we collected the samples, it rained heavily during the collection week. The rain storm could have diluted some of the nutrients in the pond. For example, land use does not affect the quality of the water. The pH was also different because it depends on the age of the pond. The older the pond is, the higher the pH, because the more macrophytes there are in the pond. For the Soluble Reactive Phosphorus test, a weak correlation is visible because plants use up all of the SRP; for testing purposes there was hardly any SRP left. Maybe, if a different form of phosphorus was to be tested, the results would correlate with the hypothesis. For example, Professor Stanley Dodson collected data displaying that total phosphorous correlates strongly with lawns. A reading of 0.737628 indicates that the land use of lawns affected the level of total phosphorous. This experiment is still important because an over abundance of nutrients affects many of organisms that live in ponds and if this happened to the ponds this could also happen to our lakes which is where we get our water from.

**Conclusion**

Our results did not support our hypothesis. The reason being is because our data concluded that land use actually had no affect on the nutrients that were tested. Resulting in further test needing to be done.

**Acknowlegements**

We would like to thank Robert Bohanan and Brian Asen for giving us the opportunity of participating in the Summer Science Institute Program. Also to Stanley Dodson, Christine Donahoe, and Kenneth Forshay, we thank you for giving us the necessary guidance through this experiment. To Owen Smith and Grant Goettl for their help and support. Finally to our SSI participants for giving us their moral support at all times.

**Works Cited**

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5. "Nitrogen- Understanding Lake Data". Wisconsin Department of Natural Resources., 2006.


9. 3_Renwick.pdf#search='purpose%20artificial%20ponds'>


Appendix

A.

Ammonium Results:

The Ammonium test was performed on July 24, 2006. 20 pond samples were tested to find the concentration. Pond eight and 20 were tested twice to see how accurate the spectrophotometer was. This Chart shows how concentrated the pond was. The $r^2$ shows how close we were, which proofs the accuracy of the test. If the $r^2$ is lower than .99, then the test was not accurate and it had to be done again.

B.

SRP Test:

The SRP test was performed on July 20, 2006. 20 pond samples around the Madison were tested also. Pond number ten and 25 were tested twice to show the accuracy of the spectrophotometer.

C.

Nitrate Test:

This test was performed on July 26, 2006. 20 pond samples around the Madison area were collected. Pond number seven and 19 were tested twice to check the accuracy of the spectrophotometer.

D.

pH Test:

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</table>

This test was performed on July 10, 2006 to the 20 ponds that were collected. This test shows how acidic the pond water is for each pond.

*Water has to be neutral for it to be drinkable (6-9). Lower than 6 is acidic, and greater than 9 is alkiline.
E.

**Specific Conductance:**

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This test was performed on July 17, 2006. The 20 pond samples were tested to see how well the ponds conduct electricity.

*There is no specific level on where the pond water should be.