Properties of vernal ponds and their effect on the density of green algae (*Oophila amlystomatis*) in spotted salamander (*Ambystoma maculatum*) egg masses

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Abstract

Amphibian populations are threatened and declining in many parts of the world and have been since the 1970s. Salamanders in particular are important mid-level predators that can provide direct and indirect biotic controls of species diversity and ecosystem processes. They also provide an important service to humans as cost-effective and readily quantifiable metrics of ecosystem health and integrity. Since salamanders are important ecosystem health indicators, steps should be taken to ensure their survival. One species in particular, *Ambystoma maculatum* or the spotted salamander has a unique symbiotic relationship with green algae, *Oophila amlystomatis*. The algae provide oxygen to the developing salamanders, helping them to grow quickly and improving post-hatching survival rates. The algae, on the other hand, use the CO_2 and nitrogenous waste produced by the salamander embryos. Lab studies have shown that increased light, warmer water and increased partial pressure of oxygen benefit the algae, which in turn help the salamanders. In this study, the environmental variables of water temperature, dissolved oxygen (DO), pH, conductivity, algae eater density, canopy cover and aquatic vegetation were compared to the density of algae found in salamander egg cells in different vernal ponds. Results showed that none of the above environmental factors had a significant relationship with algae density in eggs in the different vernal ponds. Tests showed however that density of algae in egg cells was significantly different in some ponds as compared to others, which suggests that either other environmental factors have more of an influence on algal density, or there is a complex combination of factors that need to be considered.

Introduction

The symbiotic relationship between unicellular green algae (*Oophila amlystomatis*) and eggs of the spotted salamander (*Ambystoma maculatum)* was first discussed at length by Gilbert (1942). Green algae thrives mainly in the inner envelopes of the salamander's egg, which gives the egg cell a green appearance if the algae is thick enough (Gilbert 1942). It is believed that unicellular algae invade an egg from the water the egg mass is in early on in the development of the embryos. It then makes its way into all three envelopes of the egg, outer, middle and inner envelope (Marco and Blaustein 2000). According to more recent research, the algae can also invade the cells and tissues of the salamander embryos (Kerney *et al* 2011).

Research has shown that these algae speed up the development of the salamander embryos and reduce egg mortality, while the algae actually benefit from the increased carbon dioxide (CO_2) and nitrogenous waste produced by the embryos. Therefore, it was concluded that

the algae-egg relationship was in fact a symbiotic one (Gilbert 1944; Kerney *et al* 2011). It is hypothesized that the algae supply oxygen to salamander embryos to aid in development. Oxygen is important for embryos; partial pressure of oxygen was found to strongly affect the rate of development of the embryo, the time to hatching, and the developmental stage of the hatchling in spotted salamanders (Mills and Barnhart 1999). If the amount of oxygen is too low, hatchlings could suffer developmental abnormalities or post-hatching survival rates could be reduced (Mills and Barnhart 1999). Even though oxygen is vital, it is actually very difficult to get oxygen to the innermost embryos because the eggs are packed very tightly in the jelly (Pinder and Friet 1994). Yet, most of the time the innermost embryos hatch at the same time and at the same developmental level as the outmost eggs (Pinder and Freit 1994). This can become possible when the algae present in the egg envelope provide oxygen to the innermost embryos to aid in development (Pinder and Friet 1994).

Given this evidence, it is apparent that algae are vital for the development of salamander embryos. Studies have been conducted to analyze the various factors that can increase algal growth in the egg mass, providing more oxygen to developing salamander embryos (Gilbert 1942; Gilbert 1944; Pinder and Friet 1994; Mills and Barnhart 1999). It has been found that warmer water correlates with increased algal growth in egg masses. Warmer water also makes the egg cells less viscous, so more algae can pass through the cell readily (Gilbert 1942). Also, if eggs are keep under dark conditions versus conditions with either continuous light or light and dark cycles, less algae develops within the egg mass (Gilbert 1942).

These conditions have been tested in the laboratory, but few field studies of factors such as temperature and light availability and the subsequent effect of the amount of algae in the egg masses have been conducted. As discussed above, algae can lead to greater hatching rates and

later developmental stages before hatching. It is important to understand which environmental factors can increase algal abundance in spotted salamander eggs because amphibians such as salamanders are on the decline (Stuart *et al* 2004). Amphibians are more threatened and declining more rapidly than both birds and mammals, and this decline has existed since as early at the 1970s in the western United States, Puerto Rico, and northeastern Australia. (Stuart et al 2004). Given these rapid declines in amphibian species, it is important now more than ever to determine which specific environmental factors can influence the survival of a various amphibians. In the case of spotted salamanders, the symbiotic relationship with green algae can help with embryo development and eventually hatchling survival, so determining which environmental variables can help algae help the spotted salamanders is important.

The hypothesis being tested includes examining how the environmental variables of overhead canopy cover, the temperature, DO, pH and conductivity of water the egg masses are laid in, and the density of algae eaters in the ponds relate to the abundance of algae in spotted salamander eggs. First, the overhead canopy cover influences the amount of light available to the algae which influences algae growth (Jennings *et al* 1999). I expect increased canopy cover would decrease algae growth. Second, the temperature of the water that the spotted salamander eggs grow in has been shown to affect the density of algae in lab settings, but not in field experiments (Gilbert 1942). I would expect that warmer water increases algal growth. Thirdly, dissolved oxygen can be related to algae abundance. Algae give off oxygen when they photosynthesize, increasing DO, so the more algae, the higher the DO during the day (Pinder and Freit 1994). Algae are related to pH because of their photosynthetic properties. Algae photosynthesis can raise pH by increasing the level of hydroxide, so a higher pH can be correlated with increase amounts of algae (Moss 1973). The conductivity of the water depends

on ions present in the water. Algae growth is aided by minerals like iron, nitrogen and phosphorus which also contribute to conductivity, so I expect that increased conductivity would be correlated with increased algae density (EPA 2012). Moreover, the density of algae eaters is important because an increase in algae eater presence in the water would theoretically reduce the amount of algae available to live within the egg masses. And lastly, the interaction of these environmental factors may be important, because one factor may depend on the other to affect algal density, or two factors combined may have a greater effect than either alone. Therefore I predict that canopy cover and algae eaters will be negatively correlated with density of algae in salamander egg masses while warmer water temperatures will be positively correlated with the density of algae in the eggs. Higher DO, pH and conductivity would also be positively correlated with algae density in the vernal ponds and therefore in the egg cells.

Materials and Methods:

Site selection and environmental measures

This research was conducted at the University of Notre Dame Environmental Research Center (UNDERC) located in the Upper Peninsula of Michigan. The property consists of a northern mesic forest of around 6150 acres with 30 lakes and bogs making up an additional 1350 acres. Spotted salamander egg masses were collected randomly from vernal ponds on the UNDERC property throughout the months of May and June 2012. Thirty-five egg masses total were collected by hand from twelve different vernal ponds (See Figure 1). The eggs were then taken back to lab and stored in glass containers until pictures were taken. Environmental properties such as temperature, DO, pH and conductivity were recorded at each of the twelve vernal ponds on the same day within three hours, for consistency. Temperature, in degrees

Celsius, and dissolved oxygen were measured and recorded at the surface of the water in each pond using a YSI 55 model DO meter. pH was measured and recorded at the surface in each of the ponds using a Hanna Instruments model pHep 5 pH meter. Conductivity was measured and recorded in the vernal ponds about 6 inches under the surface of the water with a Hanna Instruments HI 9033 multi-range conductivity meter. Lastly, overhead canopy cover was measured using a Spherical Densiometer, Model-C (Callam County 2012).

Algae Eater Density

The density of algae eaters in each pond was quantified by taking 3 random Integrated Tow samples at each pond. The volume of water the tow sampled was calculated by using the diameter of the tow opening and the length the tow was cast in the water. This was then used in the formula $\pi*(\text{radius})^2*\text{length}$. 50 mL of each tow sample was taken to the lab, and three subsamples of two milliliters each were examined from the initial 50 mL. Since various zooplankton are the main algae eaters in vernal ponds, the number of *Cyclopoids, Chydroids, Daphnia, Naupli, Chironomids, Calnoids, Ostracoda* and mosquito larvae were counted in each 2 mL subsample. The three subsample zooplankton quantities were then totaled to get total count per 6 mL. This quantity was then multiplied by 50 (the 50 mL total sample), divided by 6 (the 6 mL subsample volume) and finally divided by tow volume to find the density of zooplankton in the volume of water I initially sampled. The density of each of the seven zooplankton species and the mosquito larvae were calculated for each vernal pond in order to represent algae eater density within the vernal ponds.

Analysis of Density of Algae in Egg Masses

The jelly matrix of each egg mass varied in clarity and often obscured the green algae within, so to determine the density of algae in each of the egg masses, I took a picture of

individual egg cells. From each of the 35 egg masses, three individual egg cells were removed using a pipette and placed on glass slide. This slide was put on a white piece of paper under a Leica EZ4 microscope with all microscope lights on in order to maintain a consistent light source. A close-up picture was taken of the slide using a Canon PowerShot SD1400 14.1 megapixel digital camera on the auto setting, without flash. These pictures were then converted to an HSB (hue, saturation and brightness) stack using the program ImageJ. The 'brightness' stack was used, so the lower the pixel value found, the darker the image was and the higher the algae density. To get an average value of pixel intensity within an egg cell, three randomly placed 80x80 pixel circles were put in each egg cell. The intensity of the pixels was determined within each circle to give the average pixel intensity.

Statistical Analysis

All data were analyzed with Systat 13 (Systat Software Inc.) and initially checked for normality using the Shapiro-Wilkes normality test and histograms. It was determined that dissolved oxygen, conductivity and algae eater density values were not normally distributed so these variables were transformed using a natural log function. An initial Pearson correlation was run to determine how strongly correlated the environmental measures were to each other. Regressions were then run with algae density as the dependent variable and the various environmental measures such as temperature, DO, pH, conductivity, canopy cover and algae eater density as independent variables. P-values were considered significant if values are $\alpha=0.05$ or less. To determine if the twelve vernal ponds were different from each other in terms of algae density, an ANOVA and Tukey's Honesty-Significant Difference post-hoc test were run. Finally, to determine if the difference in algae might be due to presence or absence of aquatic vegetation, a two sample Student's t-test was run.

Results

The correlation initially run to determine the relationship between the various environmental factors showed that in my data DO was positively correlated to temperature and negatively correlated to conductivity and canopy cover (See Table 1 and Figure 2). Canopy cover was also strongly positively correlated to conductivity and negatively correlated to temperature, while conductivity was negatively correlated with temperature (See Table 1 and Figure 2). pH was also positively correlated with conductivity (See Table 1 and Figure 2).

The regressions run showed no significant relationship between algae density in salamander eggs and temperature of the water ($p=0.132$, $R^2=0.212$), DO ($p=0.886$, $R^2=0.002$), pH ($p=.9256$, $R^2=0.0009$), conductivity ($p=0.445$, $R^2=0.059$) and algae eater density ($p=0.913$, $R^2=0.001$) (Figure 3). Density of algae in the salamander eggs did show a significant relationship with canopy cover ($p=0.035$, $R^2=0.373$); as percent canopy cover increased, algae density increased (Figure 3). Since this was not what was expected, more tests were run to determine what other factors may be influencing algae density more than canopy cover, or in addition to canopy cover. When a multiple linear regression was run with percent canopy cover and number of samples per pond, it was found that number of samples had more of an influence on algae density in eggs than canopy cover. If more samples from more ponds were collected, a true relationship between algae density in eggs and canopy cover might have been seen.

The results of ANOVA revealed that algae density was different across ponds ($p=0.000002$, $F_{(11, 93)}= 5.21$) (Figure 4). Tukey's post-hoc test revealed that algal density in salamander eggs in vernal pond WD1 was significantly different from algal density in salamander eggs in vernal ponds WD3, C, 18, N, 5 and 2 ($p= 0.015, 0.002, 0.007, 0.002, 0.002,$

0.00007 respectively). In addition, algal density in salamander eggs in vernal pond PL was also significantly different from that in vernal pond 2 (p= 0.011).

The result of the two sample t-test revealed that there was a difference in density of algae in salamander eggs between ponds with or without aquatic vegetation (p=0.036, t= -2.37) if variance between presence and absence of algae was considered, not pooled variance (Figure 5). This relationship was not significant however when a multi-linear regression was run with number of samples per pond and aquatic vegetation presence against density of algae in salamander eggs. Number of samples had a stronger influence on algal density than aquatic vegetation (p=0.04 vs p=0.114 for aquatic vegetation), with higher algal density in ponds with less samples (Figure 6).

Discussion

The results of the present study do not support the hypothesis that warmer water, higher DO, pH and conductivity would show a positive relationship with algae density in salamander eggs. In fact, these environmental measures did not show any type of relationship with density of algae in egg cells of salamanders. Amount of light able to reach the pond was also not related to algae density in salamander eggs when number of samples was considered. Gilbert (1942) found in laboratory experiments that continuous light, or at least light and dark cycles, resulted in a rich algae growth within the egg cell envelopes when compared with eggs placed in the dark. The fact the canopy cover, which would control the amount of light available to reach the egg masses, did not significantly affect algae density when number of samples was taken into account suggests that light may not be the most important factor for algae growth in salamander eggs in the field. The fact that most canopy cover does not produce black light conditions as in a

lab but simply variability in the amount of sunlight able to reach the pond could also explain why there was not as definitive a relationship between light and algae growth in the field.

Gilbert (1942) also suggested that warmer water would increase algae growth because warmer water leads to an increased rate of development of the salamander embryo. Since the algae depend on the nitrogenous waste and CO_2 produced by the embryo, it makes sense that algae growth would increase with increased embryo development (Gilbert 1942). It is also believed that increased temperature makes the eggs less viscous, allowing the algae to more easily invade the egg cell (Gilbert 1942). The degree of temperature differences needed to obtain these results was not reported in Gilbert, however this experiment shows no positive correlation between algae density and temperature of the vernal ponds (Gilbert 1942). It could be the case that the temperature of the ponds did not vary enough to affect algae growth within the eggs, or other unknown confounding variables affect algae density.

Mills and Barnhart (1999) showed that hatching success, hatchling development and rate of development were positively correlated with partial pressure of oxygen. This increased rate of development would encourage algal growth in the eggs, which would produce more oxygen and increase DO (Gilbert 1942; Mills and Barnhart 1999). This was not found in the vernal ponds in this experiment; however it could be the case that more environmental factors such as temperature, aquatic vegetation or surface area of the pond are affecting DO. To see if algae density and DO are more closely related, lab experiments with egg masses of different algal densities and their respective DO measurements would have to be conducted.

The lack of relationship between algae density in salamander eggs and pH and conductivity could also be the result of confounding variables that were not measured. Many things could affect pH and conductivity, including ions from rainwater or soil (EPA 2012). It

could just be the case that pH and conductivity are not as strongly related to algae density as they are to other factors or combination of factors. In terms of aquatic vegetation, the initial significant relationship between aquatic vegetation and algal density was not present when number of samples collected per pond was looked at in conjunction with aquatic vegetation. This was the same case with percent canopy cover and algal density, which suggests that sample size and number of samples is really the issue here. If more samples were collected and aquatic vegetation was better quantified as emergent vegetation percentage, a stronger comparison between density of algae in salamander eggs and aquatic vegetation could be possible.

The lack of relationship between algae density in salamander eggs and algae eaters in the vernal ponds could be explained by the process of algal invasion into the egg cell. Immediately after the eggs are laid, the egg masses are generally small and condense. During the first couple of days after the eggs are laid, the gel matrix around the eggs swell as water is absorbed, and it probably during this process that the algae invade the egg cells (Marco and Blaustein 2000). Unfortunately the warm spring of 2012 allowed for the deposition of eggs earlier than normal and the egg masses collected in May and June were already hatched. This means that the algae were already inside the egg cell where algae eaters could predate upon it. Therefore, the density of algae eaters tested in the vernal ponds at a time when the algae were already in the eggs might not reflex the true relationship between the two variables. It would be better to test algae eater density immediately after the egg deposition in the vernal pond because it is these algae eaters that could really affect how much algae can enter the salamander eggs.

The fact that none of these environmental variables had a significant relationship with density of algae in salamander eggs did not hide the fact that the density of the algae seemed to be different in the different ponds, and the ANOVA confirmed this (Figure 4). So if temperature,

DO, pH, conductivity, canopy cover and algae eater density differences between the vernal ponds did not account for the difference in algae density in eggs, then it must be some other biotic factor, abiotic factor, or combination of both that creates the difference between ponds. The Pearson correlation confirmed some environmental factors are correlated to each other, which means the combination of factors could affect algae density but perhaps not individual factors alone as tested here (Figure 2). The difference between ponds is not very large however. The post-hoc from the ANOVA revealed that most of the ponds were not significantly different from each other and only a few really stuck out in comparison to others. So it may be the case that variables like light and warmer water positively influence algae density in eggs in the lab, yet in the field at UNDERC, enough confounding variables and complex environmental interactions affect the algae and eggs that such clear-cut relationships are not evident.

One important thing to note about this study is that the egg masses were collected post-hatching. It has been suggested that algae density is greatest in the last stages of embryo development, so it should represent of the greatest extent of the symbiotic relationship if most egg masses were collected post hatching (Gilbert 1942). Yet it has been shown that the algae failed to grow and thrive when the salamander embryos are not present and may start to die as the jelly mass degrades (Gilbert 1944). This is a problem because there was no way of telling exactly how long the egg masses had been without embryos. A better experiment would be to collect egg masses from vernal with embryos still present and measure algae density immediately after they hatch in the lab, but measure all the previously mentioned environmental factors at the ponds at the time of collection.

While previous lab studies have shown that density of algae increases with increased temperature of water, light availability and partial pressure of oxygen, which in turns benefits

salamander larvae, this field study of such factors did not support such claims. This suggests that there are multiple factors and combination of factors in vernal ponds that can affect algae density in salamander eggs (Gilbert 1942; Gilbert 1944; Pinder and Friet 1994; Mills and Barnhart 1999). It is important to remember that lab studies do not transition perfectly to the unpredictable field environment and that more work needs to be done to understand exactly what environmental factors can benefit the algae-salamander embryos interactions. If we do this, we can work to protect the declining amphibians that are vitally important to ecosystems (Davic and Welsh Jr 2004; Stuart *et al* 2004). Further studies should include more test sites, collection of egg masses that are not already hatched, quantitative measurement of emergent vegetation and partial pressure of oxygen measurements. Such information could help tease apart what effects algae density and subsequently spotted salamander populations.

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Tables

Table 1. Pearson Correlation Matrix values. These values range from 0 (no correlation) to +/- 1 (perfect correlation). Over 0.8 is a strong correlation and values between 0.4 and 0.7 are correlated.

	Temperature	**DO**	**pH**	**Conductivity**	**Canopy Cover**
Temperature	1.00				
DO	0.315	1.00			
pH	-0.414	0.159	1.00		
Conductivity	-0.552	-0.679	0.471	1.00	
Canopy Cover	-0.738	-0.697	0.338	0.786	1.00

Figures

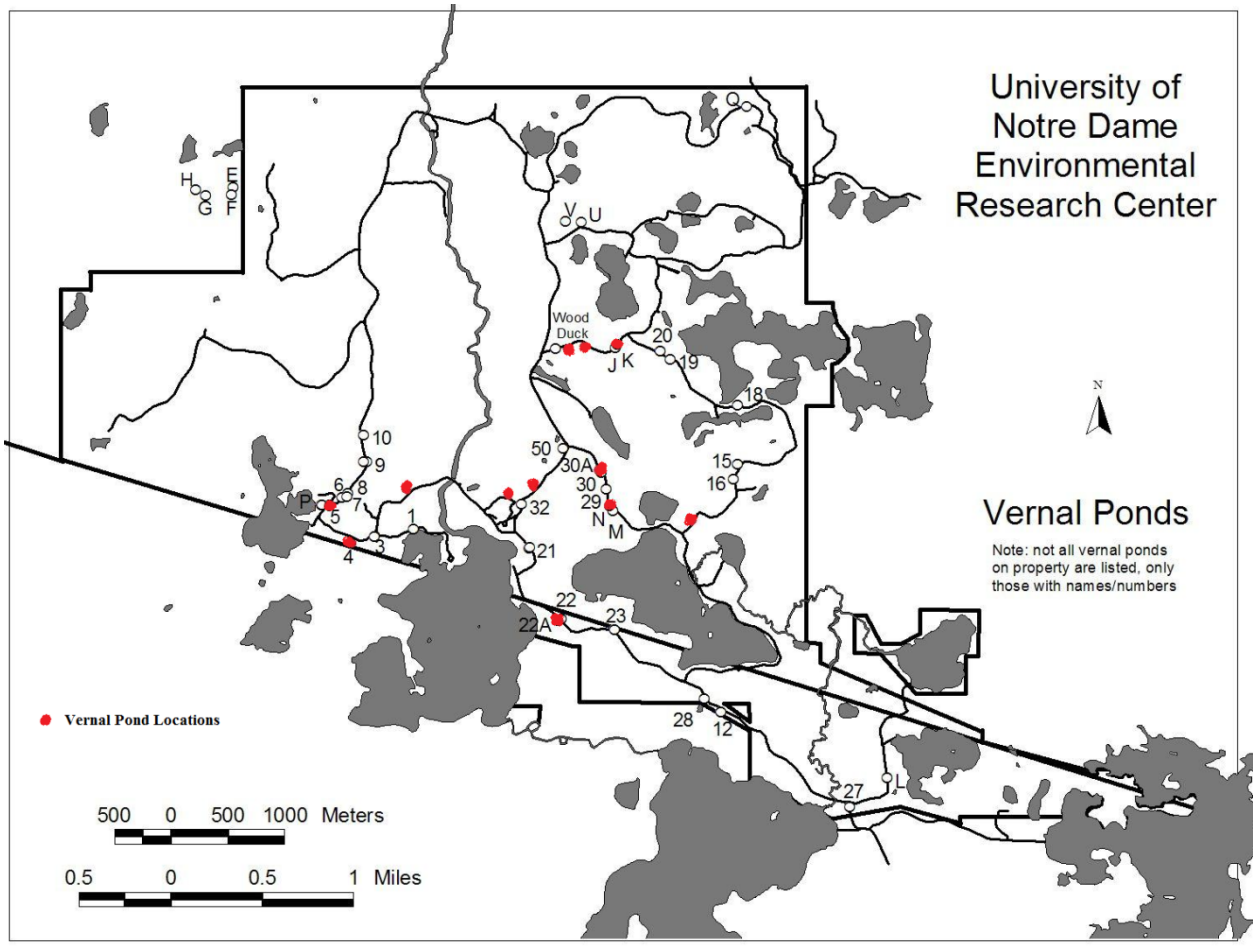

Figure 1. Map of vernal ponds on UNDERC property. The red dots indicate the location of the twelve vernal ponds were salamander egg masses were collected and various environmental measures were recorded.

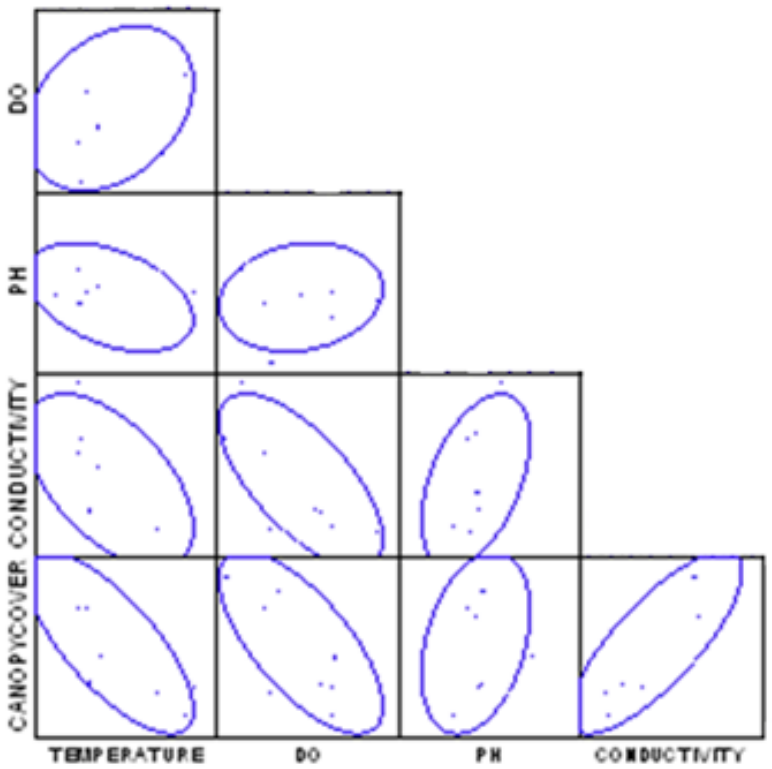

Figure 2. Correlations of Environmental Measures. A visual representation of Table 1, showing the correlations between the environmental variables tested.

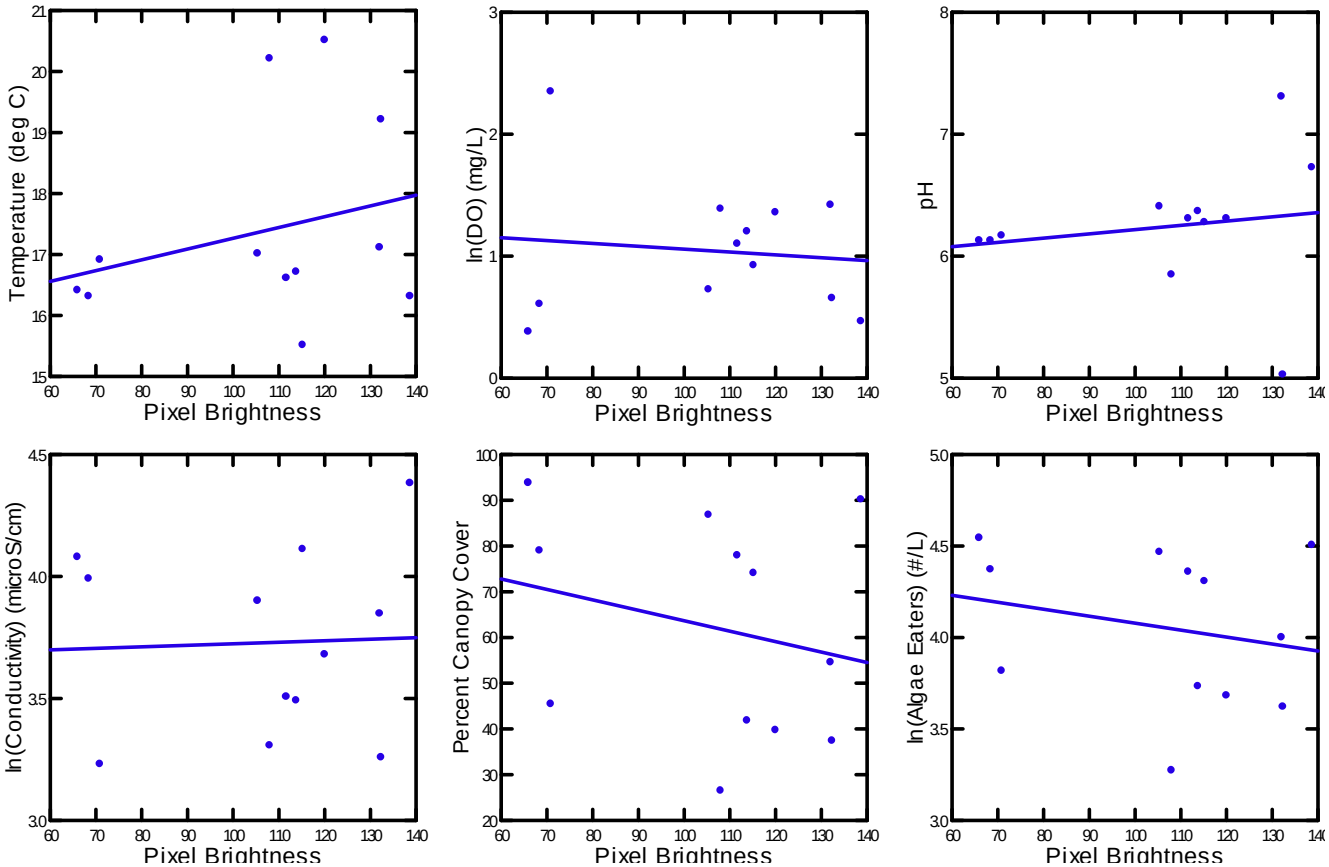

Figure 3. Relationship of measured environmental factors and density of algae in salamander eggs. The only significant relationship was an increase in algae density with an increase in percent of canopy cover ($p=0.035$, $R^2=0.373$).

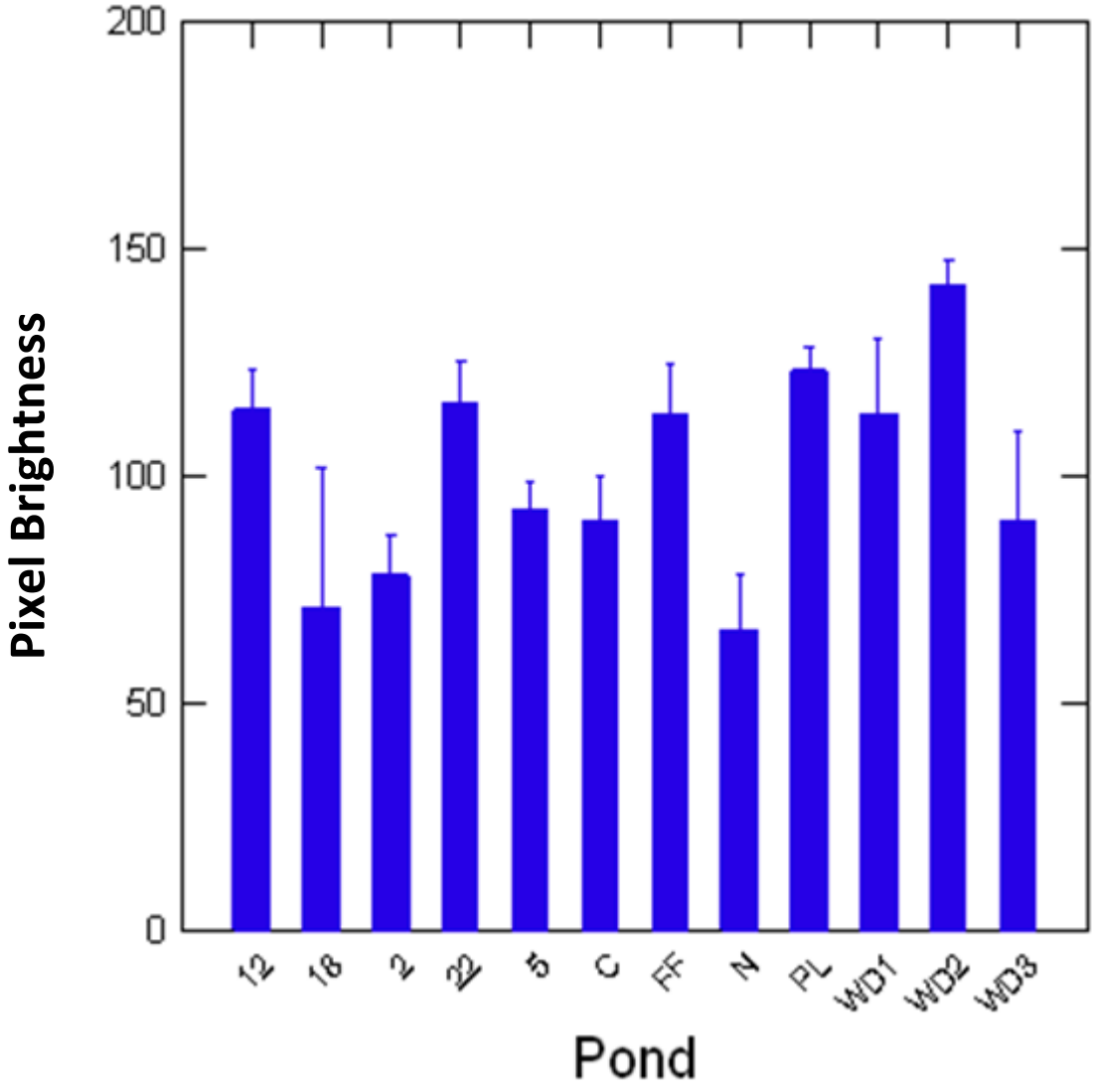

Figure 4. The density of algae in salamander egg masses in the twelve vernal ponds sampled with standard error. Decreased pixel brightness means increased algae density. The algal density differed significantly between ponds ($p=0.000002$, $F_{(11, 93)}= 5.21$).

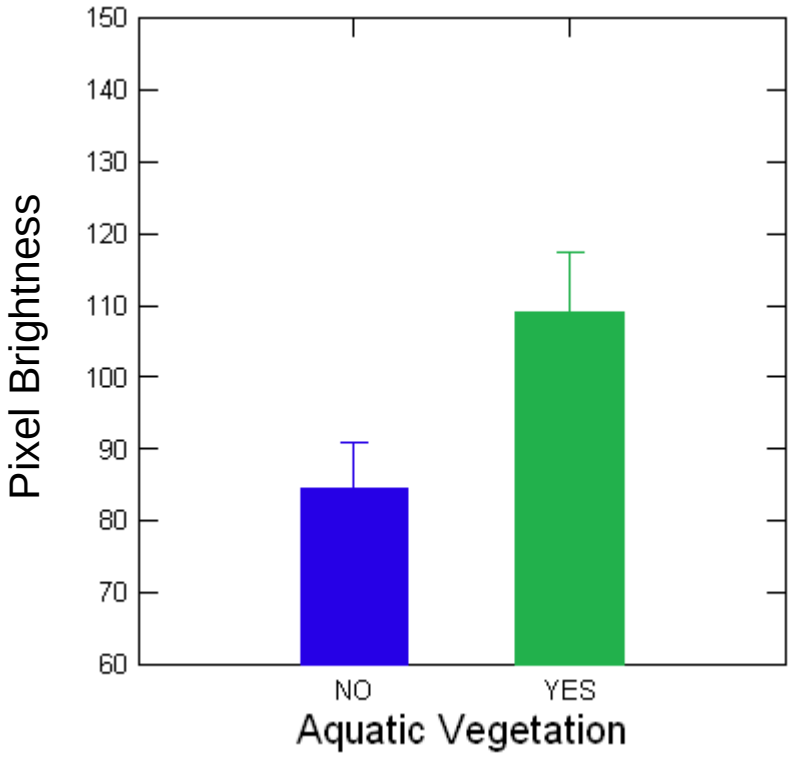

Figure 5. Visual representation of difference in algae density in ponds that do and do not have aquatic vegetation. Aquatic vegetation presence effects algae density in salamander eggs ($p=0.036$, $t=-2.37$). This relationship was not apparent however when number of samples per pond was run with aquatic vegetation in a multi-linear regression ($p=0.114$, $F_{(1,9)}= 3.06$).

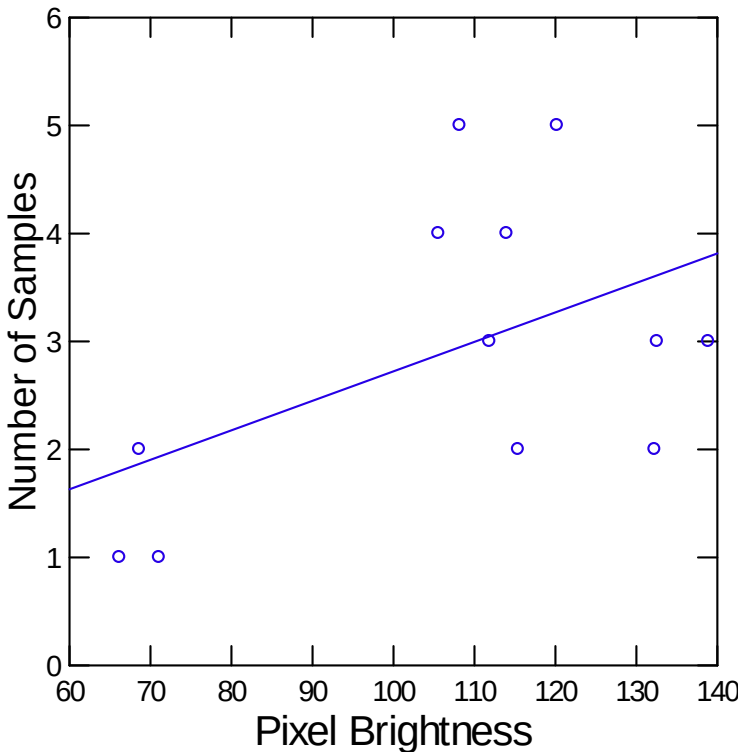

Figure 6. Relationship of Number of Samples and Pixel Brightness. Number of samples was significantly related to pixel intensity ($p=0.028$, $F_{(1,10)}=6.612$). Lower pixel brightness (increased algae density) was associated with decreased number of samples.