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HABITAT SELECTION ACROSS A TEMPERATURE AND NUTRIENT GRADIENT BY  
COPE'S GREY TREEFROG (*HYLA CHRYSOSCELIS*) AND THE MOSQUITO, *CULEX*  
*RESTUANS*

A Thesis:  
Presented in partial fulfillment of requirements  
for the degree of Master of Science  
in the Department of Biology  
The University of Mississippi

by

Sarah C. McNamara

May 2020

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## ABSTRACT

Habitat selection by pond breeding amphibians and insects is often critical for larval survival, performance, and resulting parental fitness, since there is typically little parental care beyond oviposition site choice. Numerous axes of patch quality have been identified and quantified, but species with varied life histories may differently shift perception of habitat quality as changes in global climate increase water temperatures and rates of eutrophication. Three outdoor mesocosm experiments were conducted using two species with complex life cycles and very different life histories; *Hyla chrysoscelis*, or Cope's Gray tree frog, and the mosquito (*Culex restuans*). Two mesocosm experiments were conducted to test the oviposition preference of each species by establishing gradients of temperature and nutrients in a completely crossed design. Additionally, a third mesocosm experiment testing larval performance of *H. chrysoscelis* was conducted by measuring frog metamorph body metrics of individuals reared in each temperature and nutrient combination. The temperature and nutrient treatments showed no significant effect in any of the three experiments, but each experiment had significant covariate predictors. The temperature difference from control pools in each block had a significant positive linear relationship with the number of *H. chrysoscelis* eggs laid per mesocosm, and metamorph mass increased with higher nitrate levels. *C. restuans* laid more egg rafts in mesocosms with higher levels of nitrate, and nitrate was driven significantly by temperature. Both species choose to oviposit in treatments that may ultimately benefit their offspring through increase rate of development and survival. The environmental variables that influence oviposition choice and offspring performance are crucial to understanding species distributions, abundances, and

community structure as global warming and eutrophication affect the quality of freshwater systems.

## ACKNOWLEDGMENTS

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# **CHAPTER I: OVIPOSITION PREFERENCES AND LARVAL DEVELOPMENT OF COPE'S GRAY TREE FROG (*HYLA CHRYSOSCELIS*) ACROSS A TEMPERATURE AND NUTRIENT GRADIENT**

## ***Introduction***

Habitat selection is a process where organisms disperse and non-randomly colonize habitat patches based upon perceived quality, and it largely explains the unequal distribution of individuals within an ecosystem (Fretwell and Lucas 1969, Pulliam and Danielson 1991, Binckley and Resetarits 2005). The chosen habitat patch should ideally be where individuals will have the highest fitness, but species vary in their capabilities to assess and actively choose a habitat (Fretwell and Lucas 1969, Resetarits 2005). Habitat quality may be influenced by a wide range of biotic factors such as predator presence and competition, as well as abiotic factors, such as temperature and desiccation risk (Seale 1982, Shurin and Allen 2001, Binckley and Resetarits 2002, Kurdíková et al. 2011, Pintar and Resetarits 2017a).

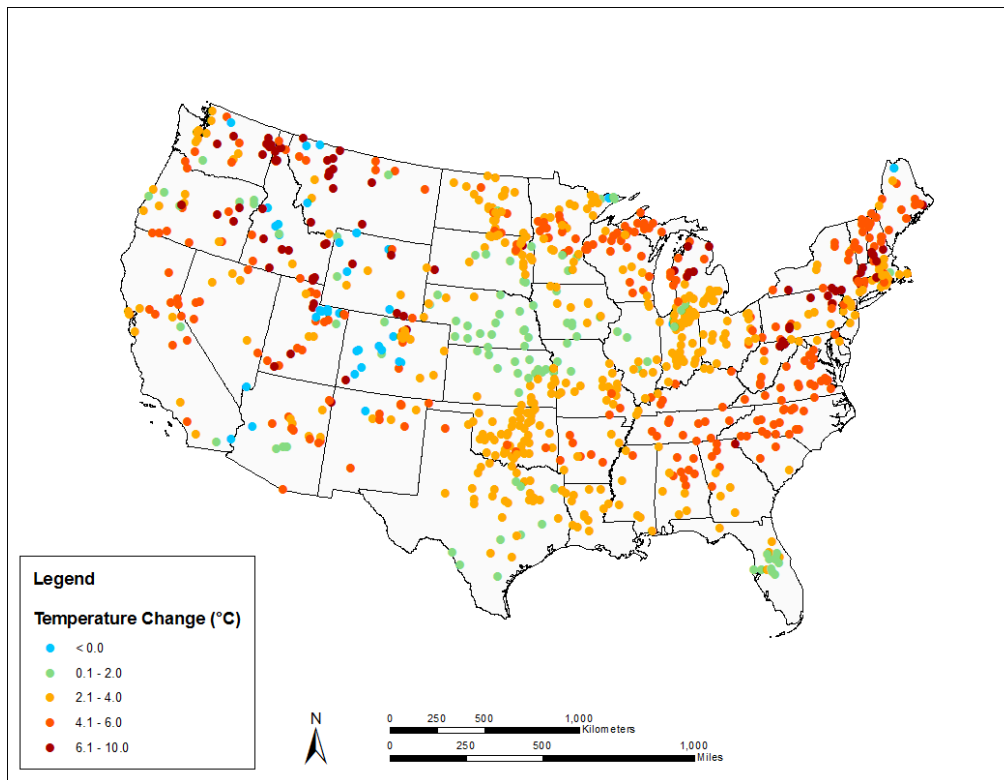
Habitat selection by pond breeding amphibians is of vital importance since there is typically little parental care for the offspring beyond oviposition site choice (Resetarits and Wilbur 1989, Eveland et al. 2016). The quality of the patch chosen can ultimately determine offspring performance and subsequent adult fitness. Given variation in patches, habitat selection theory suggests that females should choose a location that will maximize offspring performance (Fretwell and Lucas 1969, Pulliam and Danielson 1991). If a certain biotic or abiotic factor influences larval mortality or survival, then females may show a response in their oviposition habitat selection (Rieger et al. 2004). For example, the hydrophilid beetle

*Tropisternus lateralis* will oviposit less often in habitat patches where predators are present and increase oviposition as nutrients become more abundant in the predator-free locations (Binckley and Resetarits 2008).

The qualities of a habitat that are perceived as optimal can vary with time, especially in a changing environment (Rosenzweig, 1991). The abiotic conditions of habitat patches may be more important during the initial stages of habitat selection, as individuals need to be able to survive, and ideally, thrive in their environment (Kraft et al. 2015, Logan et al. 2015).

Temperature and resource availability are two of the most important variables in freshwater habitats that determine habitat quality and play a major role during the colonization and distribution of organisms (Magnuson et al. 1979, Burgmer et al. 2007, Moran et al. 2010, Pintar et al. 2018). Additionally, water temperature and nutrients can change significantly within a habitat due to global climate change.

Global temperatures are expected to increase 0.3–4.8 °C over the next century (IPCC 2013). Temperatures in freshwater systems are highly correlated with regional air temperature and the average projected water temperature increase is 3.5 °C in lakes 2–30 m deep on the continental United States by 2085, with some lakes in the U.S. projected to increase as much as 9.5°C (Fig. 1.1) (Butcher et al. 2017). Shallow lentic habitats are particularly vulnerable to temperature and desiccation because of their large surface-to-volume ratios (Scheffer et al. 1993, Adrian et al. 2009).



**Figure 1.1.** Lake map from Butcher et al., 2017. Simulated changes by 2085 of summer mean weekly average temperatures above the thermocline for >800 lakes in the continental US.

Increased water temperatures can cause negative effects on individual species, biomass, production, and community composition (Biro et al. 2007, IPCC 2007, Vincent 2009, Gardner et al. 2011). For example, warmer water can decrease water quality by favoring floating plants which suppress macrophytes below the surface and can increase turbidity (Feuchtmayr et al. 2009). This, along with external nutrient loading from agricultural runoff, can increase the amount of phytoplankton, decrease periphyton levels, and reduce dissolved oxygen (Feuchtmayr et al. 2009, Vincent 2009). This input of nutrients and increased temperature amplify the effects by increasing internal nutrient loading (Stewart et al. 2013, Guignard et al. 2017). Consequently, these conditions allow for the dominance of a few tolerant species, decreasing biodiversity (Qin et al. 2013).

Temperatures directly influence organisms through changes in respiration, metabolism and energy demand which can affect growth, survival, and reproduction rates (Gillooly et al. 2001, Brown et al. 2004, Köhler et al. 2011). Because of this, increased temperatures can cause negative effects, especially for amphibians, which rely on environmental temperatures to regulate their body temperature. Adults searching for a suitable habitat or a mate may encounter an increased risk of desiccation and a decrease in performance as temperatures rise (Mitchell and Bergmann 2016). Additionally, the offspring of many species with complex life cycles may be negatively impacted by increasing water temperatures, as aquatic systems are necessary for reproduction and for the larval stages of their lifecycle when they have limited dispersal abilities (Jaeger 1970, Blaustein et al. 1994, Pounds 2001). Due to these limitations, it would be expected that ovipositing adults select habitat patches that will provide their offspring with the highest chance of survival to metamorphosis.

*Hyla chrysoscelis* (Cope's gray treefrog) is a common tree frog in the Southeastern United States that breeds from April through August. Many *Hyla* species show oviposition responses to abiotic and biotic variables that often improve larval performance such as avoiding predators, optimizing pond size and age, and nutrient level (Rieger et al. 2004, Pintar and Resetarits 2017b, Resetarits et al. 2018, Magee 2019). However, there is less information from field experiments about the influence of temperature, nutrients and their interaction on adult oviposition and larval performance. To address these questions, two outdoor mesocosm experiments were conducted to investigate potential temperature and nutrient thresholds in the oviposition site choice, and larval performance of *H. chrysoscelis*.

## ***Methods***

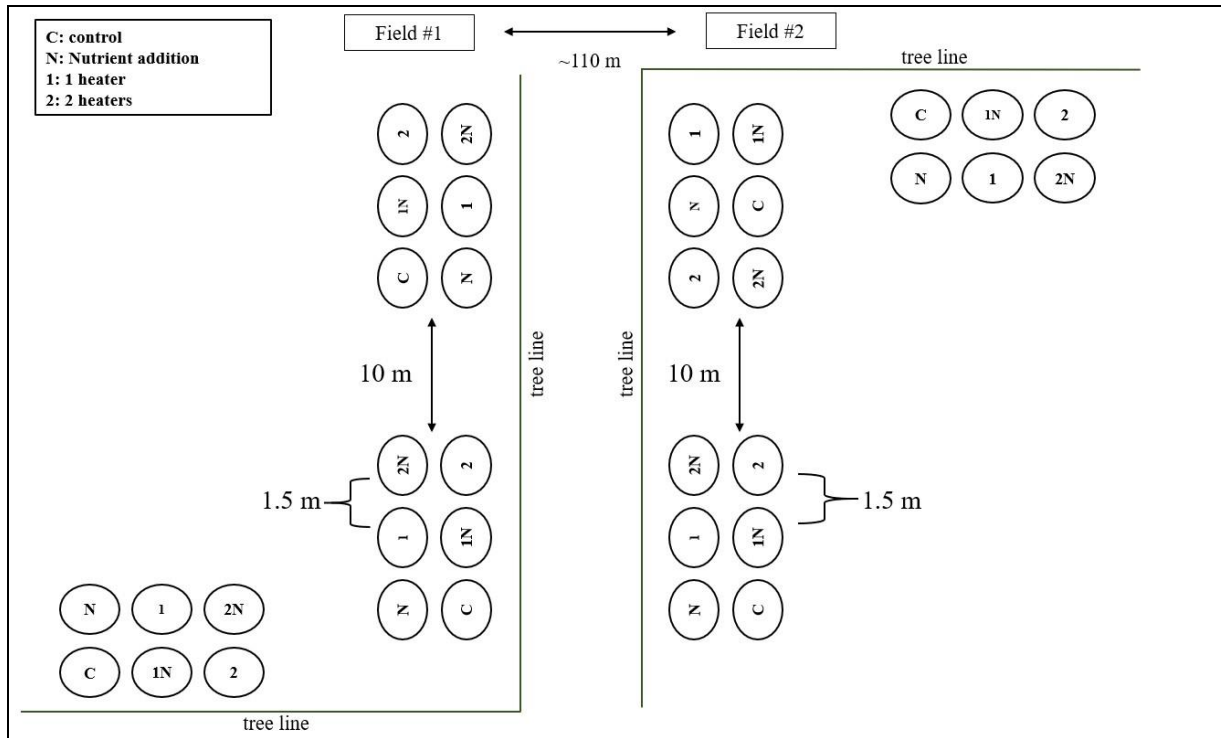
### *Study Site*

The oviposition and development studies were conducted at the University of Mississippi Field Station (UMFS) in Lafayette County, MS. The UMFS is 318 hectares with over 200 ponds, several streams, mixed hardwood and conifer trees, wetlands, and abundant wildlife.

### *Oviposition Experimental Design*

To test the effect of water temperature and eutrophication on *H. chrysoscelis* oviposition, an outdoor mesocosm experiment was conducted from May 17 until August 26, 2018.

Mesocosms were set up on two fields that are approximately 110 m apart; both of which were >30 m from the nearest pond. A total of 36 mesocosms (1200 L cattle tanks) were arranged into six blocks and set up in a 3×2 factorial design (see Fig. 1.2), crossing three levels of temperature (0T=control, 1T=1 aquarium heater; 2T = 2 aquarium heaters) with two levels of nutrients (0N=control and 1N=added nitrogen/phosphorus reagent). Each pool within a block was 1.5 m apart and blocks were 10 m apart. Blocks 1–2 were filled on 10 May, blocks 3–4 on 12 May, and blocks 5–6 on 15 May. All pools were open to colonization on 18 May once treatments were established.



**Figure 1.2.** *Hyla chrysoscelis* oviposition experimental design. To test the oviposition preferences of *H. chrysoscelis*, a 3×2 factorial design was established that crossed three levels of temperature and two levels of nutrients, using a total of six blocks.

Eheim Jager Aquarium Thermostat Heaters (250 W and 300 W) were placed vertically near the bottom of all mesocosms and were set to their maximum levels, except for in control treatments, where they were left off. Nutrient treatments received 100 ml of a nitrogen/phosphorus reagent ( $80 \mu\text{g L}^{-1} \text{P}$ ,  $1500 \mu\text{g L}^{-1} \text{N}$ ) once at the beginning of the experiment to mimic seasonal input to eutrophic lakes in the surrounding area (U.S. Environmental Protection Agency 2000). Additionally, all mesocosms received 1 kg of well-mixed, predominantly hardwood, leaf litter to establish a nutrient base, were fit with standpipes to maintain water levels and were tightly fit with fiberglass screen lids ( $1.3 \times 1.13 \text{ mm}^2$  openings) that were pushed down below the water surface to allow for *H. chrysoscelis* oviposition and efficient egg collection.

Once weekly, a YSI Pro Plus Multiparameter Instrument was used to analyze temperature, dissolved oxygen and pH, but dissolved oxygen measurements could not be used due to sensor malfunction. Every two weeks, chlorophyll levels were tested in situ using a TD-700 Laboratory Fluorometer and phosphate levels were tested using a HANNA Low Range HI-96713 Phosphate Portable Photometer. Ammonium was tested until 29 June, and nitrate was tested from 30 June until the end of the experiment.

Each morning, all mesocosms were checked for eggs. When present, eggs were collected, put into individually labeled containers and taken back to the lab, photographed and counted using ImageJ, and released into a fishless pond at the field station (Schneider et al. 2012, Bohenek and Resetarits 2017). *H. chrysosealis* is the main anuran species observed ovipositing in mesocosms at UMFS and this was confirmed by surveying calling males several nights throughout the experiment and rearing eggs from the experiment until metamorphosis.

#### *Development Experimental Design*

An outdoor rearing experiment was established to test the effect of water temperature and nutrients on the growth and development rate of *H. chrysosealis* tadpoles. Eggs were removed the morning after oviposition from mesocosms from the previously described experiment, hatched in the lab, and groups of 100 individual hatchlings were randomly selected to be reared while the rest were placed in a fishless pond at the field station. Each group of 100 individuals were randomly split into two groups, with 50 being placed in the treatment they were originally laid in and 50 being placed into control treatments to monitor and test for variation between clutches.

Each group was reared in a 300 L plastic wading pool that had 0.1 kg of leaf litter from the surrounding area and was left to age for three to four days. Nutrient treatments received one

addition of 3 ml nitrogen/phosphorus reagent ( $80 \mu\text{g L}^{-1} \text{P}$ ,  $1500 \mu\text{g L}^{-1} \text{N}$ ) to reflect eutrophic lakes from the surrounding area (U.S. Environmental Protection Agency 2000). Each morning, pools were checked and metamorphs were taken back to the lab where mass and days to metamorphosis were recorded. A photo was taken of each individual and used to measure snout vent length (SVL) using ImageJ before each was released at the field station (Schneider et al. 2012). Similar to the oviposition study, temperature, pH, nitrate levels and dissolved oxygen were measured every 2–4 days using a YSI Pro Plus Multiparameter Instrument but the dissolved oxygen measurements cannot be used due to sensor malfunction after the second week. Once per week, chlorophyll was measured in situ using a TD-700 Laboratory Fluorometer, and once every two weeks, phosphate levels were tested using a HANNA Low Range HI-96713 Phosphate Portable Photometer.

Overall, there were five replicates of the unheated treatment with nutrients, four replicates of control treatments, and two replicates of all heated treatments, due to limited outdoor access to electricity (Table 1.1). One replicate of the unheated treatment with nutrients was eliminated due to the presence of many large *Pantala flavescens* larvae that resulted in the death of all tadpoles. Three treatments were set up on 25 May and all additional replicates were set up when eggs from needed treatment and/or outdoor electricity access were available. Any development mesocosms that had tadpoles by 20 October were all taken down and any remaining tadpoles were counted and considered in the survival analysis.



**Table 1.1.** Number of *Hyla chrysoscelis* tadpoles used in the development experiment in each treatment, and their corresponding controls.

Treatment	# mesocosms	# individuals
0T-0N	4	200
0T-1N	4	200
-control	4	200
1T-0N	2	100
-control	2	100
1T-1N	2	100
-control	2	100
2T-0N	2	100
-control	2	100
2T-1N	2	100
-control	2	100

### *Oviposition Analyses*

Temperature was used in calculations in two different ways: as a categorical variable with number of heaters as each level (temperature: 0, 1, or 2 heaters) and nightly within-block temperature differentials for all mesocosms (temperature differential). For temperature differentials, the average of the two mesocosms without heaters served as the base temperature (temperature differential=0), which was subtracted from the average nightly temperature of all other mesocosms in that block. Testing with temperature differential may capture more accurate data than categorical number of heaters, and helps account for spatial and temporal variation in air temperature, which contributed to temperature variation throughout the summer. Nutrients were included in analyses as a categorical variable (nutrients or no nutrients).

The data used are from nights when there was oviposition and all analyses were done in R-studio version 1.2.5033. To test the effect of treatment, hits per active block night were analyzed, which included the number of nights a pool had eggs when there was oviposition activity in any pool within that block. This was completed using a linear mixed effects ANOVA

fit by maximum likelihood (using the Satterthwaite method for degrees of freedom) with type III sums of squares and arcsine square-root transformed data. A similar mixed model was used to test the effect of treatment on mean deposition, i.e. the mean number of eggs being laid in each pool per active block night using log transformed data. These models were fit using the lmer function from the lme4 package (Bates, Mächler, Bolker, & Walker, 2015). Both analyses used temperature as a categorical variable and block as a random factor and were followed with a Dunnett's test comparing Least-Squares Means to determine treatment differences from the control (Dunnett 1955). All analyses originally included the treatment interaction of temperature and nutrients but was highly insignificant in all cases and the interaction was dropped.

Additionally, simple regression was used to test the effect of temperature differentials rather than categorical temperature on mean deposition, i.e. the number of eggs being laid when there was activity. Similar analyses were done to examine if there were effects of some physico-chemical parameters on the number of eggs laid. These were done using the lm() function in the stats package. This included linear regressions for six weeks of data measuring ammonium (NH<sub>4</sub>, mg/L) (square-root transformed) from the beginning of the project up until 28 June, then testing nitrate (NO<sub>3</sub>, mg/L) (log-transformed) until the end of the project. Linear regressions testing chlorophyll-a (FSU) (square-root transformed) beginning on 9 June until then end of the project, as well as on phosphate levels (mg/L) (square-root transformed) using bi-weekly data beginning on 24 June.

#### *Development Analyses*

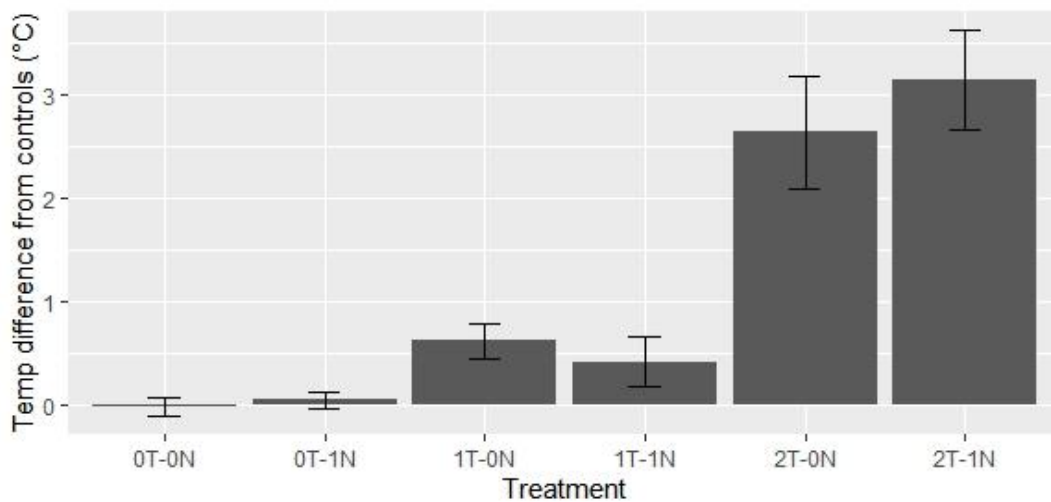
A MANOVA was used to determine the effects of treatment, phosphate, nitrate and chlorophyll on the developmental variables mass, SVL, and larval period of *H. chrysoscelis* tadpoles. A similar analysis was done to test differences in phosphate, nitrate, chlorophyll and

average temperature between treatments. These tests were done using the manova function in the stats R-package. Logistic regressions were run to analyze differences between treatments in survival and metamorphosis with pool included as a random factor (glmer function in lme4 R-package).

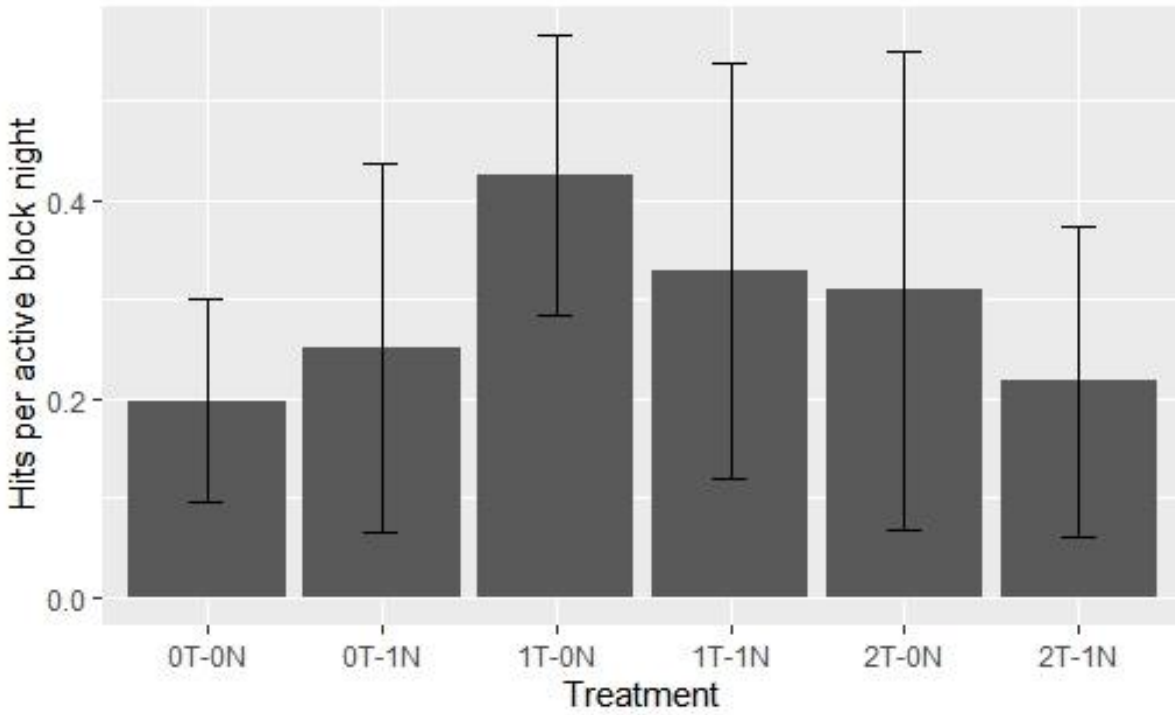
## Results

### Oviposition

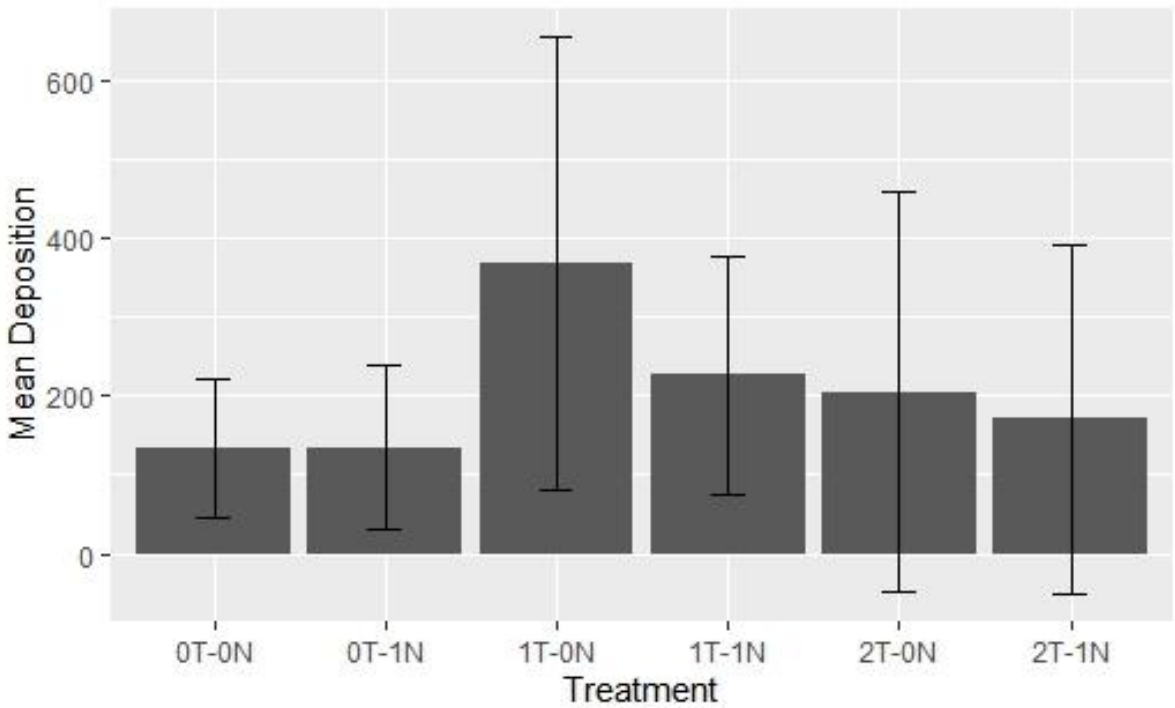
A total of 122,208 eggs were collected during the experiment. Depending on the block and individual mesocosm, there were between 11 to 27 active block nights and 0 to 13 laying events per pool. Nutrients had no significant effect in any analyses, so only temperature and physico-chemical parameters will be described. On nights when eggs were laid, nightly water temperatures ranged from 25.2–32.5°C and the mean temperature variation from controls ranged from -0.68–5.53°C (Fig. 1.3). There was no significant effect of temperature on hits per active block night ( $F_{5, 30.012}=1.45, p=0.23$ ) (Fig. 1.4) or on mean deposition ( $F_{5, 30}=0.93, p=0.47$ ) (Fig. 1.5). Pairwise comparisons of treatment means with the control using Dunnett’s procedure showed no significant differences in either test (Table 1.2).



**Figure 1.3.** Mean temperature differentials from controls for each treatment throughout the experiment (means  $\pm$  SE).



**Fig 1.4.** Frequency a treatment received eggs when there was laying activity in its block; or hits per active block night (means  $\pm$  SE).



**Figure 1.5.** Mean *H. chrysoscelis* eggs in each pool per active block night, or mean deposition (means  $\pm$  SE).

**Table 1.2.** Summary of Dunnett’s procedure comparing all treatments to the control (0T-0N) for the response variables (a) hits per active block night and (b) mean deposition.

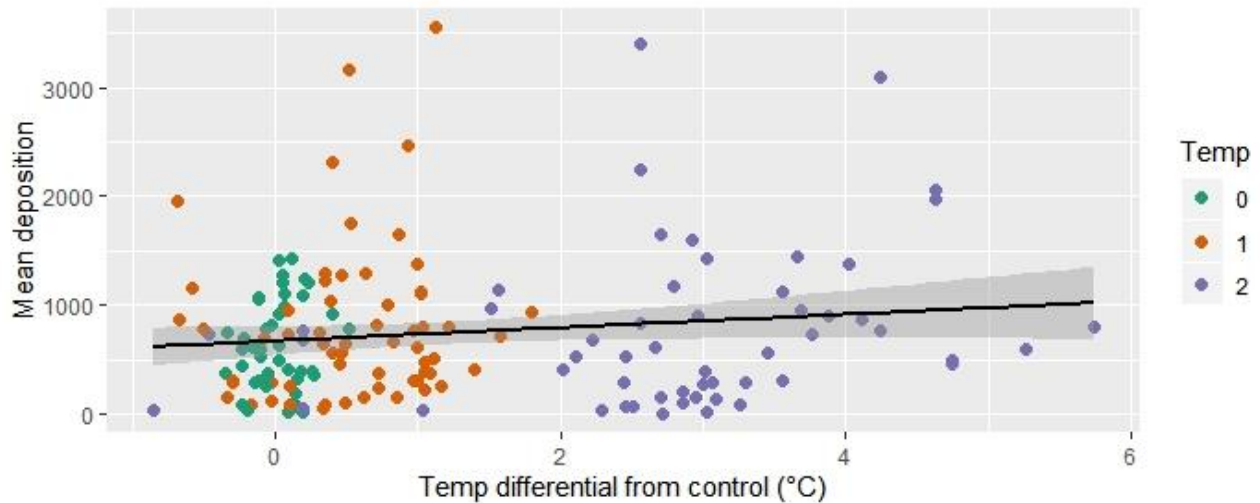
**Dunnett’s Post hoc Comparisons**

(a) Hits per active block night						
Treatment	Mean	Mean Difference	Std. Error	<i>p</i> value	95% Confidence Interval	
					Lower	Upper
0T-0N	0.1096					
0T-1N	0.1118	0.0022	0.0043	0.9810	-0.0086	0.0131
1T-0N	0.1196	0.0099	0.0043	0.0854	-0.0009	0.0208
1T-1N	0.1153	0.0057	0.0043	0.5518	-0.0052	0.0166
2T-0N	0.1143	0.0047	0.0043	0.7157	-0.0062	0.0156
2T-1N	0.1104	0.0008	0.0043	0.9999	-0.0101	0.0117

(b) Mean deposition						
Treatment	Mean	Mean Difference	Std. Error	<i>p</i> value	95% Confidence Interval	
					Lower	Upper
0T-0N	4.516					
0T-1N	4.150	-0.3657	0.9184	0.994	-2.6724	1.941
1T-0N	5.683	1.1672	0.9184	0.580	-1.1395	3.4739
1T-1N	5.194	0.6789	0.9184	0.918	-1.6279	2.9856
2T-0N	4.102	-0.4137	0.9184	0.989	-2.7205	1.8929
2T-1N	4.439	-0.0769	0.9184	1.000	-2.3837	2.2297

There was a marginal effect of temperature differential on mean egg deposition, with the number of eggs laid increasing as the temperature differential from the controls increased ( $F_{1, 157}=3.04, p=0.08$ ), although only a small proportion of the variation was explained (adjusted  $R^2=0.08$ ) (Fig. 1.6). There were no significant effects of ammonium ( $F_{1, 47}=1.05, p=0.31$ ), nitrate ( $F_{1, 80}=2.74, p=0.10$ ), chlorophyll-a ( $F_{1, 111}=0.07, p=0.79$ ), or phosphate ( $F_{1, 15}=0.64, p=0.44$ ) on where eggs were being laid.



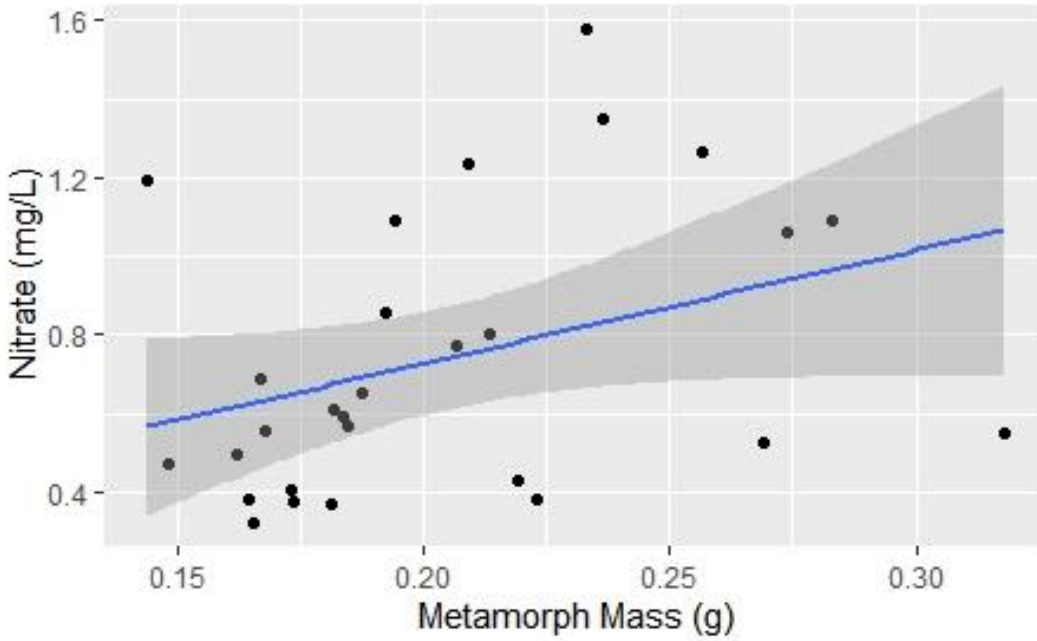
**Figure 1.6.** Temperature differential from controls had a marginal effect on mean deposition (number of eggs laid when there was laying activity), showing an increase in eggs as temperatures increased ( $F_{1, 157}=3.04, p=0.08$ ).

### *Development*

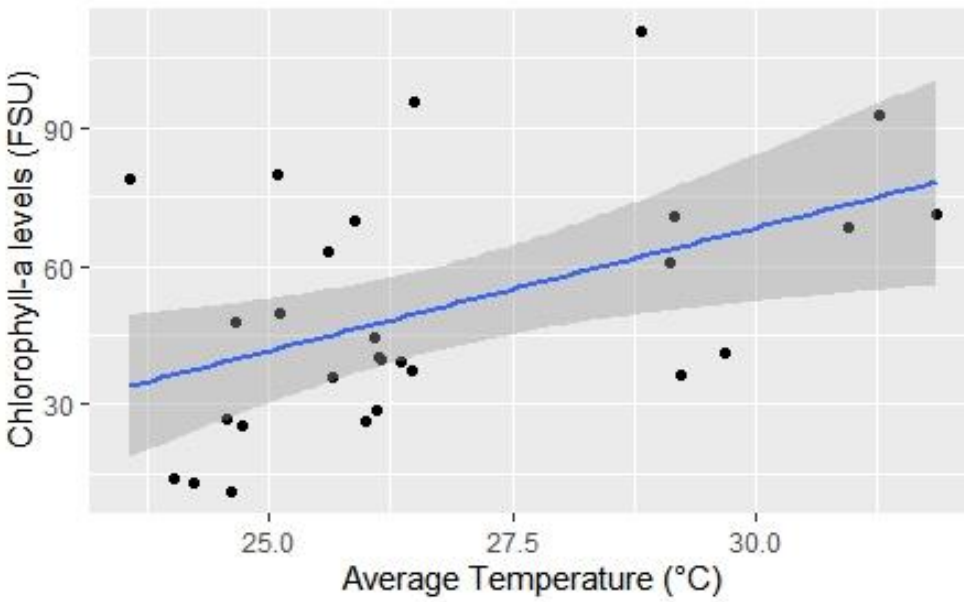
A total of 1,400 tadpoles were placed in mesocosms. The tadpoles experienced an overall survival rate of 55.2%, with 91% of those individuals completing metamorphosis. Treatment, chlorophyll, and phosphate had no significant effect on larval period, mass or SVL (Table 1.3). Nitrate levels had a marginally significant effect on metamorph mass with mass increasing as nitrate levels rise ( $F_{1,7}=4.18, p=0.08$ ) (Fig. 1.7). There was a significant difference in average temperature between heated and non-heated treatments, demonstrating that heaters effectively raised the temperature of mesocosms above the control levels (Table 1.4). Additionally, there was a significant difference in chlorophyll levels and nitrate levels between heated and non-heated treatments. A linear regression showed a positive linear relationship between temperature and chlorophyll ( $F_{1,26}=7.65, p=0.01$ ) (Fig. 1.8). Treatment had no significant effect on larval survival or metamorphosis.

**Table 1.3.** MANOVA results showing effects of treatment, nitrate, phosphate, and chlorophyll on metamorph mass, SVL and larval period. Univariate ANOVA results of water quality parameters on (a) mass, (b) SVL, and (c) larval period.

MANOVA-metamorph mass, SVL and larval period.				
	df	<i>F</i>	Wilk's $\lambda$	<i>p</i>
Treatment	5,7	0.6	0.2792	0.8646
Nitrate	1,7	2.5	0.3958	0.1697
Phosphate	1,7	0.9	0.641	0.4895
Chlorophyll	1,7	0.1	0.9328	0.9444
(a) Univariate ANOVAs-Metamorph Mass				
	Df	<i>F</i>	<i>p</i>	
Treatment	5,7	0.3251	0.8828	
Nitrate	1,7	4.1816	<b>0.0801</b>	
Phosphate	1,7	2.0786	0.1926	
Chlorophyll	1,7	0.0452	0.8377	
(b) Univariate ANOVAs-Metamorph SVL				
	Df	<i>F</i>	<i>p</i>	
Treatment	5,7	0.7493	0.6118	
Nitrate	1,7	1.6970	0.2339	
Phosphate	1,7	1.3877	0.2773	
Chlorophyll	1,7	0.0043	0.9495	
(c) Univariate ANOVAs-Larval period				
	Df	<i>F</i>	<i>p</i>	
Treatment	5,7	0.4277	0.8166	
Nitrate	1,7	0.8428	0.3891	
Phosphate	1,7	0.1316	0.7275	
Chlorophyll	1,7	0.006`	0.9399	



**Figure 1.7.** Relationship between nitrate levels and metamorph mass ( $F_{1,7}=4.18$ ,  $p=0.08$ ). The shaded area represents a 95% confidence interval.



**Figure 1.8.** Relationship between temperature and chlorophyll levels in the development mesocosms ( $F_{1,26}=7.65$ ,  $p=0.01$ ). The shaded area represents a 95% confidence interval.



**Table 1.4.** (a) MANOVA results for nitrate, phosphate, chlorophyll and average temperature between treatments, and the treatment groups heated vs. non-heated, and nutrients vs. no nutrients. (b) Univariate ANOVA results for the treatment group heated vs. non-heated. Significant values are in bold.

nitrate, phosphate, chlorophyll and temperature.				
	df	<i>F</i>	Wilk's $\lambda$	<i>p</i>
Treatment	1,14	0.96	0.7408	0.4658
Heat vs no heat	1,28	26.8	0.1892	<b>1.022e<sup>-16</sup></b>
Nutrients vs. no nutrients	1,28	0.99	0.8631	0.4304
ANOVA-heated vs non-heated				
	df	<i>F</i>	<i>p</i>	
Ave. Temp	1,28	107.44	<b>4.335e<sup>-11</sup></b>	
Nitrate	1,28	4.0391	<b>0.0542</b>	
Phosphate	1,28	0.0962	0.7587	
Chlorophyll	1,28	6.1017	<b>0.01986</b>	

### *Discussion*

Determining oviposition preference and assessing larval performance can help predict species distributions and abundances, and can allow for a better understanding of species abundances and community assembly (Binckley & Resetarits, 2007, 2008; Resetarits, 2005). Many aquatic organisms are particularly susceptible to climate change since water temperature greatly influences ectotherm metabolism (Blem et al. 1986). Therefore, increases in energy demand and locomotion may require an increase in the nutrients consumed (Abram et al. 2017). Because of the effects of warmer water and nutrient levels, we would expect adult oviposition to closely match offspring performance, as the ability of a female to assess biotic and abiotic variables in a habitat before oviposition will ultimately determine reproductive success and may influence community dynamics (Resetarits and Wilbur 1989, Resetarits 1996, Haramura 2008).

Nutrient levels did not affect where females oviposited, which has been confirmed previously (Binckley and Resetarits 2007, 2008, Resetarits et al. 2018). This result is surprising since *H. chrysoscelis* tadpoles are herbivores and an increase in nutrients is known to increase

larval performance by limiting resource competition (Leibold and Wilbur 1992). There was no effect of the temperature treatment, likely because the categorical treatments did not capture variation in the actual temperature of mesocosms. Although the range of temperatures in this study were fairly limited and nightly temperature were variable, as the temperature differentials from the control pools increased, there was a marginal, but positive linear relationship with the number of eggs laid. Higher water temperatures are known to be beneficial through an increased growth rate of amphibian larvae up until a point where the high temperature causes individuals to have smaller mass, developmental abnormalities and fatalities (Smith-Gill and Berven 1979, Petranka 1984).

Temperature and nutrient treatments had no effect on larval mass, SVL, or larval period, but there was a very marginal positive linear relationship between nitrate levels and metamorph mass. This makes sense, because nitrate promotes phytoplankton and periphyton growth which provides more nutritional resources for tadpoles to consume before metamorphosing. Higher nutrients may be initially beneficial for offspring, but high nitrate and chlorophyll levels, which increased with higher temperatures, may indicate an increase in algal blooms and decrease dissolved oxygen (Linløkken, 2019; Paerl & Huisman, 2008). Higher nutrients and algal blooms has also been associated with increasing rates of disease transmission and prevalence in aquatic habitats (Johnson, Carpenter, Ostfeld, Keesing, & Eviner, 2008; Paull & Johnson, 2018). Although the sample size from the development study is too small to be definitive, nine metamorphs that were reared in the nutrient treatments had severe malformations, while only one metamorph had a deformity out of the individuals reared in non-nutrient rich mesocosms.

Although organisms may be able to behaviorally adapt to their surroundings to decrease stress, e.g., by decreasing movement, aggregating or selecting a more favorable microhabitat,

this behavior can decrease larval growth rate and survival (Petranka et al. 1987, Lawler 1989, Skelly 1992). Offspring success and parental fitness can largely be related to the oviposition site choice of the adult female (Resetarits 1996). Frogs that have a larger body size at metamorphosis often still maintain their larger size at maturity (Smith 2016). Larger males produce calls with lower carrier frequencies which may be better at attracting females, although there are conflicting results as to whether larger *H. chrysoscelis* males truly have an advantage over smaller individuals (Godwin and Roble 1983, Morris 1989, Schrode et al. 2012). Females with larger bodies are able to carry larger clutch sizes, which increases their overall reproductive output (Ritke et al. 1990). Additionally, larger individuals are able to disperse further, which is especially important for *H. chrysoscelis* because they most often utilize temporary ponds (Searcy et al. 2018). These habitats have been decreasing in numbers as forests are cleared for anthropogenic purposes and organisms may need to disperse farther or search longer to locate an appropriate habitat patch (Dodd and Cade 1998, Williams et al. 2010).

Female *H. chrysoscelis* oviposition site selection often matches some of the most critical variables determining offspring survival, such as avoiding habitat patches containing predators, and choosing larger habitat patches (Resetarits and Wilbur 1989, Resetarits et al. 2018). Additionally, they will preferentially oviposit in patches that are not under the tree canopy, which may be indicative of ephemeral ponds that may lack predators and have increased productivity (Binckley and Resetarits 2007). These behaviors suggest that light may be a more important factor in the female site choice than temperature or nutrient levels. Since all mesocosms were predator free, the same size, and not under the tree canopy, the treatments may have all matched the most important criteria for an ovipositing female. The ranges of

temperature and nutrient variation was either not large enough or predictable enough to drive oviposition site choice in *H. chrysoscelis*.

The abiotic and biotic variables driving habitat selection are crucial to understanding species assembly, distribution and abundances. Oviposition behaviors are complex and influenced by many interacting factors. Determining what the most important variables are for the success of a species will allow us to fill gaps of information needed to model population viability and their potential responses to climate change. Learning more about the factors that are not the most crucial to species habitat selection processes is also important. Increasing water temperatures and nutrients in aquatic systems are threatening many species and ecosystems. Understanding which species are at the most and least risk of these effects from global climate change will assist in prioritizing conservation efforts and provide an overall better understanding of life history patterns.

## **CHAPTER II: OVIPOSITION PREFERENCE OF A COMMON MOSQUITO, *CULEX RESTUANS*, ACROSS A TEMPERATURE AND NUTRIENT GRADIENT**

### ***Introduction***

Habitat selection for some ovipositing insects may be especially crucial for offspring survival and fitness, while other species are known to have few preferences and tolerate a wider range of conditions in which they will oviposit. There are many factors that may cause species to be generalists when it comes to oviposition, such as having mobile or polyphagous larvae that are able to better cope with poor environments, how many and how often eggs are laid, or due to sensory limitations (Resetarits 1996, Gripenberg et al. 2010, Day 2016, Schäpers et al. 2016). For example, in the moth species *Epiphyas posvittana*, oviposition is stimulated by chemical cues when an individual is in contact with a leaf, but they do not respond to volatile cues or a range of visual cues from a distance (Foster and Howard 1998). This strategy would increase the search time for a specialized habitat, but generalist species oviposit on many different species of plants and expend less energy searching.

For most species, regardless of how specialized their habitat, there are some stimuli or cues that are the most crucial for the process to be successful. Changes in regional or global air and water temperature, precipitation and seasonality can all affect the biotic and abiotic variables in the local environment to which a species is adapted. Environmental changes due to global climate change may alter species distribution and abundance (Hart and Gotelli 2011). For species that oviposit in freshwater environments, some of the most important factors that may determine offspring survival are the availability of nutrients and water temperature, as the larvae are unable

to leave this environment, and thus need to be able to physiologically withstand these original oviposition conditions (Fretwell and Lucas 1969, Pulliam and Danielson 1991, Resetarits and Binckley 2009). Low levels of dissolved oxygen in warmer, nutrient rich systems are potentially fatal for many gilled organisms that rely on dissolved oxygen for respiration, but other aquatic species that utilize atmospheric oxygen could benefit in these environments.

Some aquatic insect larvae, such as mosquitoes, rat-tailed maggots, and water scorpions, breathe atmospheric oxygen at the surface of the water using a siphon, allowing these species to survive in habitats where low dissolved oxygen restricts the presence of other species (Dunavan 1929, Snodgrass 1959, Kour et al. 2013, Day 2016). Breeding females of these taxa will often actively choose eutrophic sites that have higher levels of nutrients, which promotes growth and development of their offspring (Blaustein and Kotler 1993, Reiskind et al. 2004). Warmer water temperatures can improve the average fitness in many species by increasing the larval development rate, leading to faster life cycles and decreasing the risk of desiccation (Strickman 1988, Blaustein and Margalit 1996, Tun-Lin et al. 2000). Many predators and competitors of aquatic insects use gills for respiration and would be unable to survive in warm, eutrophic waters, allowing herbivorous air-breathing aquatic insect populations to thrive (Johnston and Haag 1996, Blaustein et al. 1999, Grigaltchik et al. 2016).

Understanding the oviposition behaviors of mosquitoes and the interacting effects of warmer water temperature and increased nutrients in freshwater systems are crucial to gaining insight into species distributions, regulation of population abundances and an understanding of patterns in disease transmission. Mosquitoes use physical and chemical cues in their environment to detect the quality of a habitat before oviposition, with some species showing more specific preferences and others being more generalists (Bentley and Day 1989). Most mosquito species

prefer ovipositing in nutrient rich locations where larvae have an increased survival rate, emergence rate and an increase of body size (Derraik and Slaney 2005, Chaves et al. 2011, Murrell et al. 2011, Nguyen et al. 2012). Some species are known to prefer ovipositing in warmer waters, and increased temperatures may benefit offspring through an increased rate of development, though often with an increased mortality rate (Reisen 1955, Gillespie and Belton 1980, Chaves et al. 2011, Ciota et al. 2014, Bohenek et al. 2017). There is less information about the interacting effect of water temperature and nutrients on mosquito oviposition site choice.

An outdoor mesocosm experiment was conducted to test the effects of water temperature and nutrients on the oviposition of the mosquito, *Culex restuans*. *Culex restuans* is an abundant, generalist wetland mosquito in the Central and Eastern United States and an important disease vector of West Nile virus (Reeves 1956, Andreadis et al. 2001). They will oviposit in any source of standing water but prefer warmer waters and nutrient rich habitats (Reiskind and Wilson 2004, Bohenek et al. 2017). These preferences lead to an increased rate of development and higher fitness, and lower sensitivity of larvae to external changes, such as temperature (Reiskind et al. 2004, Chaves et al. 2009, 2011, Muturi et al. 2011).

## ***Methods***

### *Study Site*

This study was conducted at the University of Mississippi Field Station (UMFS) in Lafayette County, MS. The UMFS is 318 hectares with over 200 ponds, mixed forests, and abundant wildlife.

### *Experimental Design*

To test the effects of nutrients and temperature on *C. restuans* oviposition, an outdoor mesocosm experiment was conducted from 2 September to 17 October, 2018. Each array was set

up for five to eight days at a time, since mosquito oviposition begins to quickly decline as the pools age (Eveland et al. 2016). Each array consisted of six black plastic pools (30 L) crossing three levels of temperature (2T=high temp, 1T=low temp, and 0T=control) and two levels of nutrients (0N=control and 1N=added N/P reagent) ( $k=6$ ), replicated six times ( $N=6 \times 6=36$ ). Pools were 3 m apart and 3 m from a tightly covered central bait pool used to attract mosquitoes to the area. All pools contained 0.1 kg of leaf litter from the surrounding area to establish a nutrients base and had a fiberglass screen lid ( $1.3 \times 1.13 \text{ mm}^2$  openings) that was pushed down below the water surface to allow for mosquito colonization and create a barrier between the heaters and the water surface. All pools contained an aquarium heater (Eheim Jager, 300W) and on day zero, nutrient treatments received 10 ml of a nitrogen/phosphorus reagent ( $80 \mu\text{g L}^{-1} \text{ P}$ ,  $1500 \mu\text{g L}^{-1} \text{ N}$ ) intended to reflect eutrophic lakes from the nearby area (U.S. Environmental Protection Agency 2000).

Every morning, mosquito egg rafts were collected from each pool and counted. *Culex restuans* is the most abundant egg raft laying mosquito species at UMFS, accounting for 99% of mosquito egg rafts identified (Bohenek et al. 2017). This was further confirmed by identifying fourth instar larvae throughout the project. Water temperature was recorded every 15 minutes using HOBO Pendant Temperature 8K data logger. On days 1, 3, and 5 for each array, nitrate levels, dissolved oxygen and pH were recorded using a YSI Pro Plus (6050000) instrument, chlorophyll-a levels were tested in vivo using a TD-700 Laboratory Fluorometer, and phosphate levels were tested with a HANNA Low Range HI-96713 Phosphate Portable Photometer. The dissolved oxygen measurements were discarded due to sensor malfunction.

### *Data Analysis*



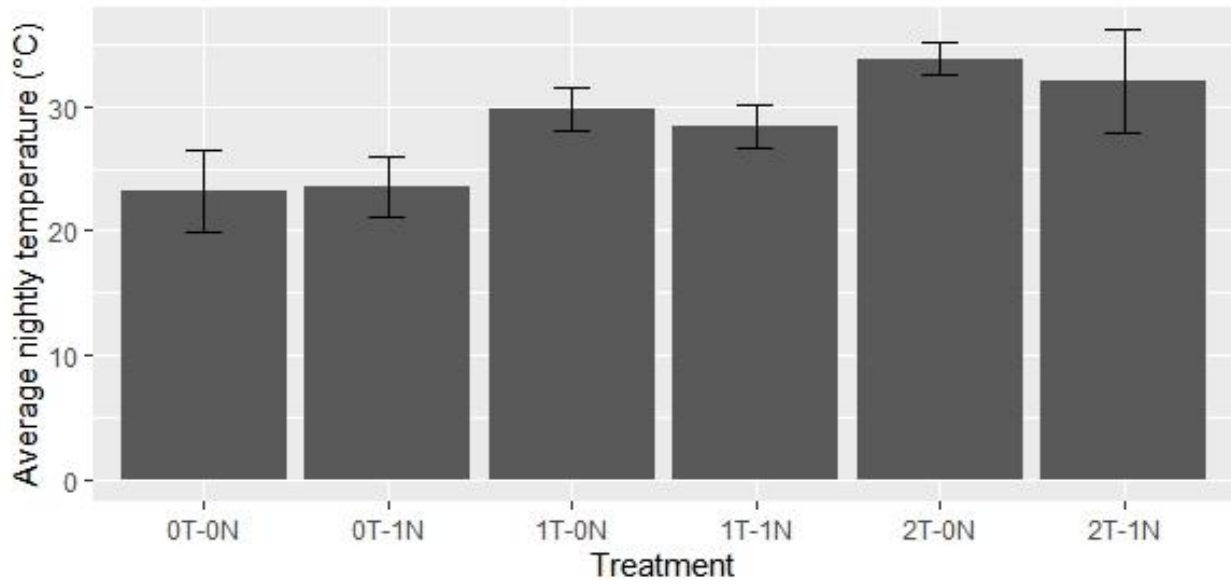
All analyses were done in R-studio version 1.2.5033. A mixed effects ANOVA fit by maximum likelihood (using the Satterthwaite method for degrees of freedom) with type III sums of squares was used to test the effects of treatment (temperature and nutrients as categorical variables) on the square root transformed total number of egg rafts per pool, with array as a random factor (lmer function, lme4 package) (Bates et al., 2015). The interaction of temperature and nutrient was initially included, but was highly insignificant and dropped from the model. When including the covariates chlorophyll-a, phosphate, and nitrate, the best model only included nitrate as a significant predictor; therefore, it was the only covariate besides array in the final model. Additionally, a simple linear regression was run to test the effect of actual temperature differential from control pools on the total number of egg rafts laid using the lm function from the stats R-package.

A leopard frog was present in one mesocosm during the experiment and resulted in much higher nitrate levels. This mesocosm is kept in the analyses as tests were conducted with and without it and the results did not change. Additionally, one mesocosm in the second array was originally established as a high temperature treatment with nutrients, but the heater malfunctioned, so this was considered a treatment without heat but with additional nutrients in the analyses.

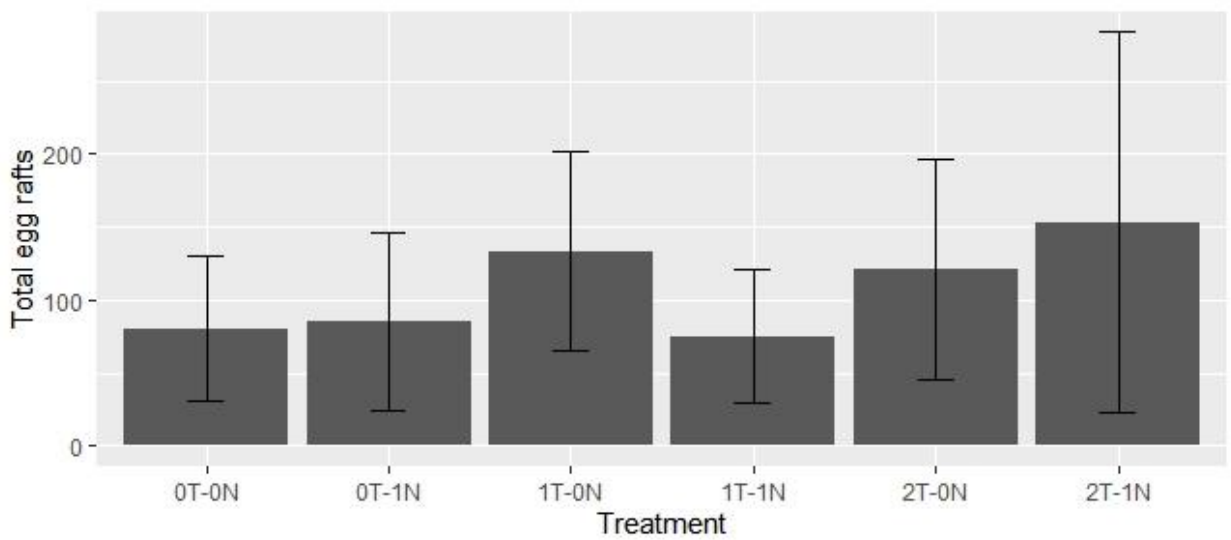
## ***Results***

A total of 3,821 egg rafts were collected during the experiment. Average nightly temperatures ranged from 14.08–37.16°C and average nightly temperature variation from controls ranged from -1.07–16.38°C (Fig. 2.1). Neither temperature treatment, nor the nutrient treatment were significant predictors of the number of egg rafts laid (Fig. 2.2), but nitrate was a significant predictor ( $F_{1,11.241}=17.55, p=0.0014$ ) (Fig. 2.3). As nitrate levels increased, the total number of egg rafts also increased, and temperature difference from controls was a significant

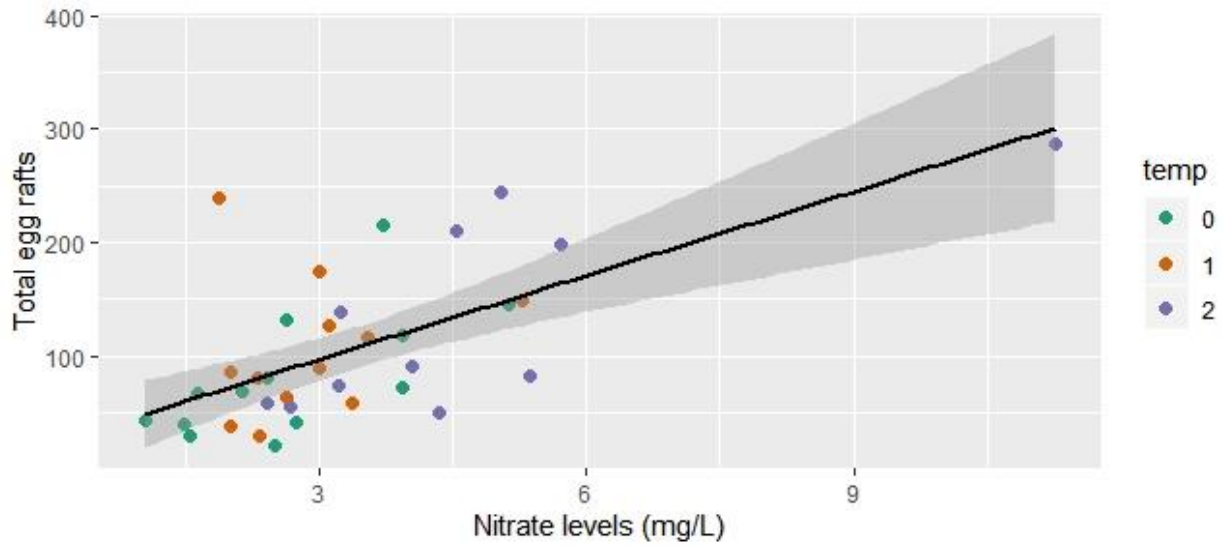
predictor of nitrate levels (log transformed nitrate) ( $F_{2,33}=7.47$ ,  $p=0.0021$ ) (Fig. 2.4) with an adjusted  $R^2$  of 0.27. The linear regression testing the effect of temperature differential from controls on the total number of egg rafts laid showed a significant effect ( $F_{1,34}=6.72$ ,  $p=0.014$ ) (Fig. 2.5).



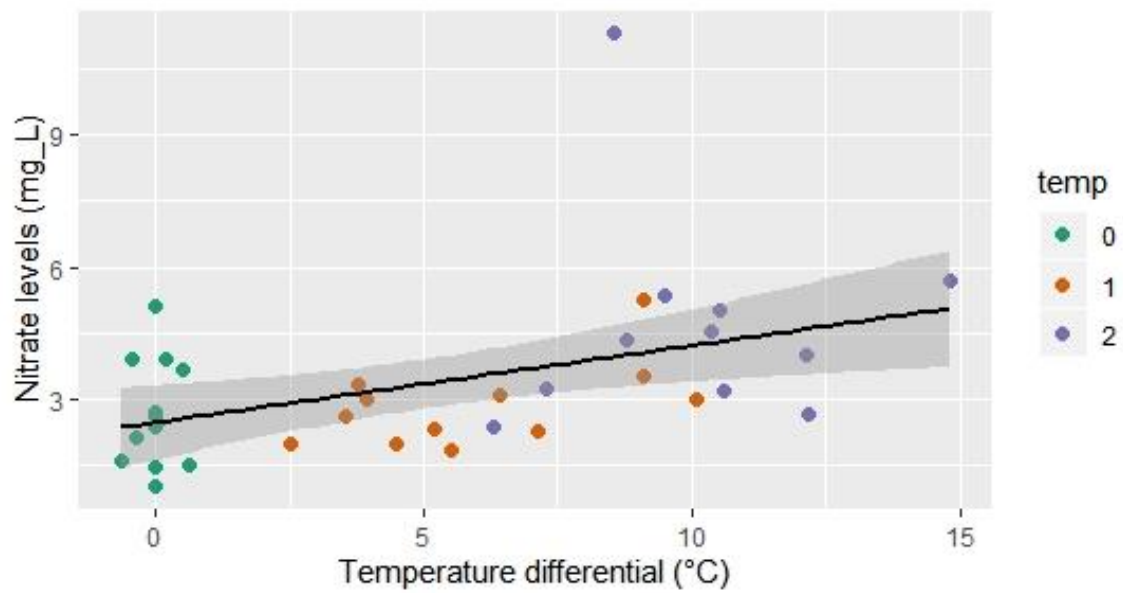
**Figure 2.1.** Average treatment temperatures throughout the experiment (means  $\pm$  SE).



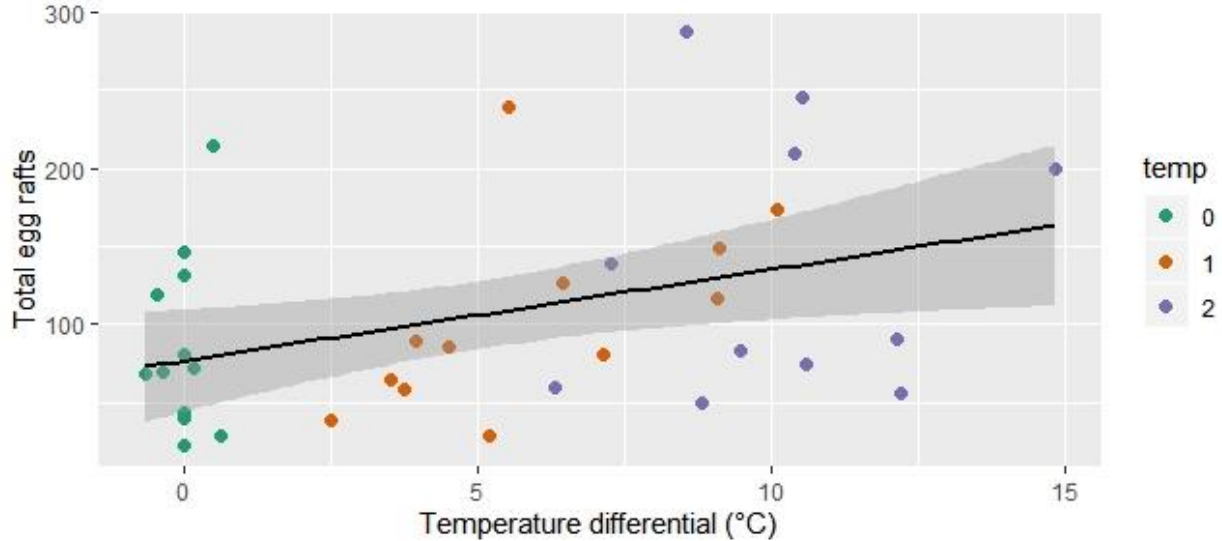
**Figure 2.2.** Total egg rafts laid in each treatment (totals  $\pm$  SE).



**Figure 2.3.** Relationship between nitrate levels and mosquito oviposition ( $F_{1,11,241}=17.55$ ,  $p=0.0014$ ). The shaded area represents a 95% confidence interval.



**Figure 2.4.** The significant relationship between temperature differentials and nitrate levels ( $F_{2,33}=7.47$ ,  $p=0.0021$ ). The shaded area represents a 95% confidence interval.



**Figure 2.5.** Relationship between temperature differentials and the total number of egg rafts laid in mesocosms ( $F_{1,34}=6.72$ ,  $p=0.014$ ). The shaded area represents a 95% confidence interval.

### Discussion

Higher water temperatures and nutrient levels can increase development rates and could potentially benefit *C. restuans* larvae, as this would lower the risk of desiccation and increase competition in their habitat (Strickman 1988, Tun-Lin et al. 2000). As seen in this study, the adult preference-offspring performance match is not always strong and clear. Both water temperature and nutrients had no effect on the oviposition preferences of *C. restuans*. There are several possible explanations for this. Although warmer water temperatures can increase larval development, it may also decrease body size or wing length at emergence due to resource limitation, as there are fewer days for feeding and growing (Carpenter 1983, Day et al. 1990). Mosquitoes that emerge with larger body sizes have more nutrient reserves to use, and larger females of many species have increased parity (Nayar and Pierce 1977, Haramis 1983, Nasci 1986, Wong et al. 2011). Another possible explanation for the lack of response to water temperature could be that water temperatures in these small habitats will typically closely mirror air temperatures. Females may be detecting air temperature to initiate egg-laying activity and if

this is the case, variation in water temperatures due to experimental manipulation may not be detected.

While there was no effect of the nutrient treatment on where eggs were laid, nitrate was a significant predictor, explaining ~31% of the variation in the number of egg rafts laid in mesocosms. Water temperature was a significant predictor of nitrate levels in the mesocosms, suggesting that the oviposition cues indicating the nutritional quality in a habitat patch may be a more important, or stronger cue to the adult than water temperature itself. This may be because water temperature in small habitats can fluctuate quickly and significantly within a day. Therefore, determining water temperature at the time of oviposition may provide an unrealistic view of the actual conditions in the habitat that offspring will experience, whereas the nutrient content is much less likely to vary dramatically during this short developmental period.

Nitrate in aquatic habitats promotes algal growth, which is the most important food source for developing mosquito larvae (Merritt et al. 1992, Kibuthu et al. 2016). Mosquito abundances and patterns of disease transmission may be impacted as the use of nitrogen on agricultural lands continues to increase (Xie and Ringler 2017). For example, the abundance of mosquito larvae increased proportionally with doses of nitrogenous fertilizers on rice fields in southern India, and the increased use of these fertilizers may be linked to major outbreaks of Japanese encephalitis, a mosquito-borne disease (Victor and Reuben 2000).

Female mosquitoes are able to respond to a large variety of oviposition cues by relying on vision, olfactory, chemosensory, and physical cues in order to find suitable oviposition habitats (Day 2016). *C. restuans* have several known oviposition preferences, such as avoiding oviposition in habitat patches with predators and a high number of conspecifics, while smaller and nutrient-rich habitats will attract oviposition (Reiskind and Wilson 2004, Eveland et al.

2016, Bohenek et al. 2017). This is seen especially at larger scales, but due to sensory limitations in flying insects in general, there are questions as to how well individuals are able to determine oviposition cues at smaller scales from habitat patches (Murlis et al. 1992, Day 2016, Montell and Zwiebel 2016, Webster and Cardé 2017, Verschut et al. 2018). This may cause individuals to misperceive the quality of one habitat patch because of oviposition cues that are coming from a patch nearby (Resetarits and Silberbush 2016, Verschut et al. 2018). Alternatively, it is possible that some environmental factors may be more difficult to assess between habitat patches, and generalist species who are flexible in oviposition site choice may demonstrate a mix of different strategies within and between populations (Wong et al. 2011).

Many mosquito species can carry vector-borne diseases and air temperature can mediate the magnitude and timing of disease spread from mosquitoes in a season and between years, with higher temperatures showing a larger spread of disease (Ruiz et al. 2010). As air temperatures increase, the subsequent increase in shallow lakes and ponds may contribute to patterns in mosquito abundance and disease transmission. Other climate variables may similarly have a significant impact on the behavior and development of mosquitoes. As the intensity of rain events increases, there is a subsequent increase in sediment and nutrient runoff into aquatic systems. Additionally, changes in seasonality may decrease mixing in shallow lakes, which creates a habitat that mosquitoes will be able to thrive in while other species will likely not be able to survive (Georgakakos et al. 2014, McCullough et al. 2019). The water temperature and nutrient levels of freshwater systems vary geographically, and understanding the oviposition behaviors of species within different regions will contribute to the current body of knowledge that may allow us to create theoretical expectations for the effects of climate change on *C. restuans* abundance and distribution.

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