The use of grey tree frog (*Hyla versicolor*) tadpoles as a biological control for eutrophication of blue-green and green algae species

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Abstract

Algal blooms are widespread due to increased fertilizer use and can affect any aquatic ecosystem. This eutrophication can lead to fish kills, trophic cascades, and dead zones. Biological controls of algae have been researched, but studies are limited to small organisms such as protozoa, bacteria, and fungi. Anurans inhabit the same wide range of aquatic systems as algae and their larval stage pre-metamorphosis depends on algae as a food source. In this experiment, I study the potential for the use of *Hyla versicolor* tadpoles as a biological control of both blue-green (*Anabanea sp*.) and green (*Chlamydomonas reinhardtii, Volvox aureus, Spirogyra, Klebsormidium*) algae species. Measurement of algal growth rate in optimal conditions determined that *Klebsormidium* had the highest eutrophication potential of my samples. *Hyla versicolor* tadpoles were fed daily these same algae samples and weight gain or loss was calculated over a week-long trial. Though this experiment was only conducted for a short time, we were able to see a trend linking high growth rate algae consumption to highest tadpole weight gain. These parallels in data suggest that *Hyla versicolor* tadpoles could be used as a biological control for algae prone to eutrophication.

Introduction

Eutrophication is the rapid depletion of oxygen in bodies of water, such as lakes and ponds, as a result of dramatic increases in algal growth rates. These algal blooms occur after dramatic increases in the nutrient loads that normally limit aquatic plant growth (Anderson, 2002). The overabundant algae eventually die, sinking to the bottom of the lake or pond where benthic organisms respire to decompose the material. The increase in respiration rates quickly depletes oxygen from the water. With lower dissolved oxygen, fish with a high oxygen demand are unable to breathe properly and fish kills can occur. Fish death produces trophic cascades, and declines in recreational fishing and overall water quality. In particularly severe cases of eutrophication, dead zones develop and no biotic activity is present within the aquatic system (Anderson, 2002).

Blue-green algae, or cyanobacteria, are a main cause of eutrophication in Wisconsin water bodies (Wisconsin DNR, 2017). Some species, when grown into blooms, can produce toxins which kill wildlife that may not yet be affected by a decrease in oxygen levels. Blue-green

algae do supply minerals to the aquatic environments, but do not generally serve as a primary food source like true algae. Green algae, the most common true algae, can also cause blooms to develop, but are more commonly found as small mats that support the developmental stages of fish, amphibians, and insects (Washington State Dep. Of Ecology, 1994). However, given optimal conditions of high nutrients, sunlight, and aeration, all algae types can overrun an aquatic system if not counterbalanced by herbivores.

Biological controls have been studied as a potential method to counteracting and controlling eutrophication. Earlier research has generally focused on using small organisms such as bacteria, fungi, and protozoa as controls (Sigee et al.,1999). These research efforts have also been concentrated on the repression of blue-green algal blooms rather than the large variety of green algae. While Connelly et al. (2008) describe how declines in tadpole populations lead to decreases in primary producer biodiversity, little is known about tadpoles' role in controlling the population sizes of primary producers. Therefore, this study will add to the limited research pool on tadpoles' potential as biological control agents for algal blooms of both blue-green and green algae species.

Prior to limb development, tadpoles consume aquatic plants, including algae. This diet provides nutrients for growth while tadpoles are still restricted to the water. As tadpoles grow into froglets with limbs, they switch to carnivorous diet of dead insects. Therefore, tadpoles act as herbivores only for approximately their first eight weeks of life (Beachy et al., 1999). Wisconsin is home to many toads (*Bufo americanus*), peepers (*Pseudacris crucifer*), and gray tree frogs (*Hyla versicolor*), which have breeding periods during the early summer months of May and June (State of WI, 2017). The summer months are also when most algal growth occurs due to increased exposure to direct sunlight (Altamirano, 2000). As *Hyla versicolor* is the latest

of the Wisconsin amphibians to breed, we selected it as our study species because the tadpoles were still in their herbivorous stage during the period when algae would be in peak bloom. Additionally, *Hyla versicolor* tadpoles are known to be tolerant of high population density and their metamorphic rate is unaffected by the nutrient enrichment present during an algal bloom making them good candidates for biological control agents (Smith and Burgett, 2012).

Tadpoles are specially adapted to feed on primary producers suspended in their aquatic habitats. Additionally, tadpoles can ingest algae particles less than $10 . \mathrm{m}$ in diameter (Wassersug, 1975). It has also been shown that tadpoles take advantage of primary production booms, such as an algal bloom, for a time of accelerated growth (Pianka, 1972). Tadpoles can also survive in a range of oxygen levels making them one of the few organisms that could survive a dramatic algal bloom. When water conditions become hypoxic, the tadpoles switch from gill usage to surface air breathing to meet the tadpole's oxygen demands (West and Burggren, 1982). The combined specialty to feed on small floating algae particles and adaptability to aquatic systems effected by eutrophication make tadpoles optimal algae consumers.

Our goal was to explore the possibility of naturally controlling or reversing eutrophication using *Hyla versicolor* tadpoles. We investigated which algal species is responsible for the highest eutrophication rates and whether tadpoles prefer this algae type. Therefore, the specific hypothesis being tested was that tadpoles will prefer the same algae that shows the most growth from an abundance of nutrients.

Methods

Preparation of Algae for Trials

We purchased small, live algae samples (23 mL) of *Spirogyra* (Spiro), *Anabanea Sp.* (Anaba), *Chlamydomonas reinhardtii* (Chlam.), and *Volvox aureus* (Volvox) from Ward's Science to ensure only one pure species would be present per sample. A mix of blue-green algae and green algae was chosen since they are the two most abundant types of algae in aquatic systems. All purchased live samples, excluding *Spirogyra,* were divided into five sub-samples (A-E) of 4.5 mL and placed into marked 250 mL beakers with 9 mL of DI water initially (Figures 1a,b,c) . Replicate sub-samples were created in case a sub-sample had to be excluded due to contamination, death, or bacteria growth. Lights were attached to the top of shelves with the beakers of algae set underneath to promote growth. Additionally, saran wrap with small holes punctured in the tops were used to cover the top of the beakers to prevent insects attracted to the lights from falling into the water (Figure 1a). We added DI water to raise the overall volume in each beaker and fed the algae a nutrient solution of 0.616 g/L of a common lawn fertilizer, Miracle Grow, approximately every four days (Rhee, 2003). Amounts of water and nutrients given to each type of algae were determined based on its rate of development, measured by absorption with a direct reading spectrophotometer (Table 1).

We collected a fifth, mixed alga sample consisting predominantly of *Klebsormidium* (Kleb) from main Pond U on the UNDERC property (Figure 3). Both this collected sample and *Spirogyra* were treated slightly differently from the other purchased samples as there was more initial algae and they did not need as strict a growing regime. The samples were each placed into

previously bleached and rinsed containers with aeration tubes connected to the air-stones placed at the bottom (Figure 2). Feedings were limited to 3 mL twice a week to continue steady growth of the both alga. All initial growth encouragements were continued until every algae species appeared to be steadily growing with the nutrient solution given (3 mL) and there was a significant volume of water total for each algae (> 250 mL) (Larson and Belovsky, 2013).

Growth Rate Trials: Algae

The *Spirogyra*, *Klebsormidium,* and healthy sub-samples of algae were placed into 1000 mL beakers by species. Each beaker had a stopper on the top with a small hole for an air hose used to aerate the algae and prevent insects from flying into the beaker. A side nozzle served to release the air pressure fin the beakers (Figure 4). To approximate the concentration of chlorophyll in a 25 mL algae sample, we measured the absorption at 675 nm with a direct reading spectrometer (Norris and Butler, 1961). Three measurements for absorption were taken per algae species and averaged to reduce sampling error. Beakers were shaken thoroughly prior to each measurement. After measuring each day, all vials were rinsed with tap water thoroughly to reduces cross contamination. Throughout the trials, algae were fed 3 mL of the 0.616 g/L nutrient solution to ensure steady growth (Larson and Belovsky, 2013). Measurements of absorbance were always completed before the addition of nutrients that could have distorted algae chlorophyll absorbance readings. I noted the water level in the beakers daily to ensure that there was not rapid evaporation and that the volume of algae to water stayed relatively consistent. Algae samples grew for one full week, and I measured their absorbance every day around noon.

Growth Rate Trials: Tadpole Consumption

On the final day of algae growth trials, the tadpole growth trials began. Eggs of *Hyla versicolor* frogs were collected during breeding season and raised together in a tank to ensure that all tadpoles were as similar as possible and at the same growing stage. Fifteen tanks were filled with 1 L of tap water and an air hose was run into each tank. All of the tank water was allowed to acclimate to room temperature for one hour as to not shock the tadpoles with varying water temperatures as they were moved to the experimental tanks. Lights were hung over all the tanks with a timer set to 12 hours of light to mimic the daylight hours outside. The tadpoles were randomly assigned to tanks with four tadpoles per tank. Each tank was randomly assigned an algae type and there were three replicates of each of the five algae types.

Before the addition of algae and daily throughout the experiment, I weighed each tadpole and recorded the total tadpole mass per tank. To measure the mass of each tadpole, I caught the tadpole using a fiberglass fly screen folded into a U as a net and weighed the tadpole with the fly screen on a scale covered in paper towels (Figure 5). The tadpole was then removed from the scale by flipping the fiberglass fly screen just over the tank so the tadpole would fall into the water and the apparatus was weighed without the tadpole in order to calculate tadpole mass. This weighing process does not injure the tadpoles if they are handled carefully during transfer as they can remain out of water for 2-4 minutes without negative effects (Browne, 2014). I replaced paper towels between each tadpole measurement. To minimize cross contamination of algae, tadpoles from different treatments were kept in different holding tanks after their weight measurements. There were five separate holding tanks for all the tadpoles assigned to each of the five algae types.

Algae was added daily to the tanks after weighing the tadpoles. To provide the tadpoles with relatively similar concentrations of algae, each tank of tadpoles received 10 mL of algae at

an absorbance level of approximately 0.04 nm. The initial absorbance was read on the direct reading spectrophotometer, after which DI water was added to the measured algae to dilute the algae concentration if necessary. Re-reading of absorbance and dilution continued until the absorbance was within +/- 0.005 of the target 0.04 absorbance.

Analysis

I used an ANCOVA to compare growth rates, measured using the absorption values, among algal specimens over time. To compare consumption rates of the different species by tadpole, I compared changes in total tadpole mass among the tanks via another ANCOVA. I preformed these analyses using SYSTAT and R while the graphical displays were created in Microsoft Excel.

Results

Algae Growth Trials

The rates of growth for each algae type were not initially normally distributed according to the Shapiro-Wilks test for normality. After a log transformation, the absorbance data were normally distributed (Shapiro-Wilk test, $p=0.8435$). The interaction between the day of measurement and the algae type significantly affected absorption readings (mean & std. error: 0.2390 +/- 0.0637, DF: 4, F: 3.7501, P-value: 0.0137). The main effects of both the algae species ($P<0.0001$) and day ($p=0.0280$) separately were also significant. Algal growth rates among specimens of the different species varied from negative to positive to approximately zero (Linear regressions, all $R^2 > 0.1231$, all $p<0.394$; Figure 3. Kleb was the only algae species with a positive slope (Figure 3). Therefore, it was considered our algae specimen with the highest

growth rate and most likely to lead to an algae bloom. Spirogyra and Anaba both had negative slopes for their growth rates, while Chlam and Volvox had slopes quite close to zero (Figure 3).

Tadpole Consumption Trials

The data of tadpole growth was normally distributed based on the Shapiro-Wilks test for normality (p=0.32278). There was no statistically significant difference between final tadpole growth for different algae types (mean & std. error: 0.0069 +/- 0.00341; ANCOVA, $F_{4,9}$: 2.0235, P-value: 0.17455). Though there was not statistical significance, we can see tadpoles in the Kleb trial trended towards more positive growth while the other trials remained almost stagnant or decreased in weight over the trial period (Figure 4). Additionally, Chlam fed tadpole groups trended towards the greatest decrease in total weight when comparing day seven to the initial weight.

Discussion

Our hypothesis that tadpoles consuming algae that had the highest growth rates would in turn have the highest weight gain was supported by the trends seen in Figures 3 and 4 as well as the statistical analysis of the algae growth rates. For this experiment, the mixed *Klebsormidium* algae and tadpole treatments fed this same alga were the clear instances of growth. The experimental tadpoles gained or lost weight, following similar trends as those seen in the growth rates of the algae they were being fed. For example, both *Anabanea* and *Spirogyra* had decreasing growth rates (Figure 3), and total tadpole weight decreased with time for all tanks containing these species. These trends show that tadpoles are selective to the consumption of algae with high growth rates suggesting them as quality candidates as biological control agents.

Though our hypothesis was supported, we did observe other unexpected results during the tadpole trials. There were a number of deaths and missing tadpoles noted starting on the third day of measurement and continuing throughout the end of the experimental week (Table 2). We did not change the total weight data as a result of these deaths or disappearances as we considered them supporting data for tadpoles' preference for the fastest growing algae that would supply them the required nutrients for growth. Tadpole deaths are fairly common as many are born with deformities and are very susceptible to disease (Cooke, 1981). However, there was an interesting concentration of deaths in the *Volvox* trial tanks (Table 2). This observation could be a result of toxins created by the algae as it continued growth into a small mat (Nwabueze, 2011). The tadpoles' sensitive skin would be highly vulnerable to any toxins created by the algae they were being fed. However, since tadpoles have a high death rate naturally we cannot be certain of this explanation for *Volvox* trial tadpole death. The missing tadpoles were particularly mysterious as there was no potential way of escape out of their tanks nor any predators with access to the tanks. Cannibalism is one possible explanation as it has been observed in some species of frog larvae (Crump, 1983). However, in *Hyla versicolor* tadpoles' cannibalism has not been seen or is extremely rare. I collected tadpoles recorded as dead preventing the other tadpoles in that tank from consuming them. These observations suggest that the behavior of *Hyla versicolor* tadpoles with their fellow hatchlings in a controlled environment deserves further study.

Errors associated with measurement techniques were present in both algae growth trials and tadpole consumption trials. With only a direct reading spectrophotometer available for absorbance reading, the accuracy of measurements for *Anabanea* and *Spirogyr*a was questionable as they grew in unevenly distributed clumps that persisted during absorbance measurements within the sample vials. Unfortunately, a more accurate technique for algae

density in water was not available because the majority of algal density calculating techniques involve killing, drying, or removing algae, techniques poorly suited for continuous study of algal growth. The tadpole weight measurements also involved a great deal of error (Figure 4) due to the small size of the individuals and the high sensitivity of the scale being used. The highly sensitive scale was necessary for recording the small weight changes of the tadpoles, but also led to potential fluctuations in weight due to water absorption into the paper towels rather than tadpole weight differences.

Given a longer trial period for both the algae growth and tadpole consumption, observed trends may have supported the initial hypothesis even more strongly. Additionally, improved measuring techniques for the algae density would decrease the errors present in this study. A replicated study with other tadpole species could determine if *Hyla versicolor* species is the only potential biological control of algae. Further studies are also required to verify if the observations of this study can be observed repetitively and are directly attributed to cannibalism or another behavioral characteristic of *Hyla versicolor* tadpoles.

The *Hyla versicolor* tadpoles consuming the Kleb algae, which had the highest growth rate, also showed the most weight gain over the week-long trial. Therefore, this experiment supports the possibility of using tadpoles as a biological control for algae. However, the use of tadpoles may not be able to be extended to all of the algae species that we tested. Toxins from the Volvox and limited nutrient availability from the algae are possible culprits for the deaths and missing tadpoles during experimentation. If the tadpoles were unable to survive because of these qualities of some of the algae, then they would not viable biological controls for those species. However, for Kleb algae blooms, at the least, tadpoles could provide an important ecosystem service of reversing and controlling algae density. An interesting follow-up study

would be to compare algal growth rates across treatments with different densities of tadpoles to see if tadpoles can cause declines in growth. Many frog species are in decline due to high predation, habitat loss, and vulnerability to disease (Vredenburg, 2010), but the use of tadpoles as a biological control could encourage more frog breeding and protection of tadpole populations for their ecosystem service. Therefore, if we increase populations and protect remaining tadpoles, we could see decreases in harmful algae blooms and increases in ecosystem health with fewer dead zones developing.

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Tables

Table 1: Schedule of Nutrient Feedings and DI water addition.

Days into Trial	Type of Algae	Water Added	Nutrients Added
1	Anaba	15 mL	3-5 drops
1	Volvox	15 mL	3-5 drops
1	Chlam	15 mL	3-5 drops
5	Anaba	25 mL	1.5 mL
5	Volvox	0 mL	3 mL
5	Chlam	25 mL	1.5 mL
9	Anaba	0 mL	3 mL
9	Volvox	0 mL	3 mL
9	Chlam	0 mL	3 mL
12	Anaba	50 mL	3 mL
12	Volvox	25 mL	3 mL
12	Chlam	25 mL	3 mL

Table 2: Instances of deaths (X) or missing (?) tadpoles for each alga type over the week long consumption trials.

Day	Anaba	Spiro	Kleb	Volvox	Chlam
0					
1					
2					
3	?	X			?
4	?	?		X	X
5				X	
6			?	X	
7					

Figures

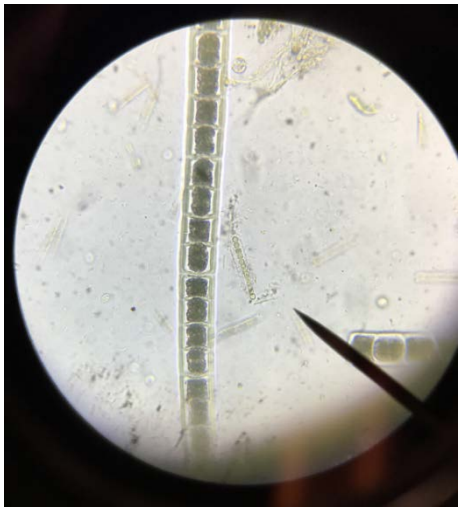

Figure 1: *Klebsormidium* sample collected from Pond U under a microscope.

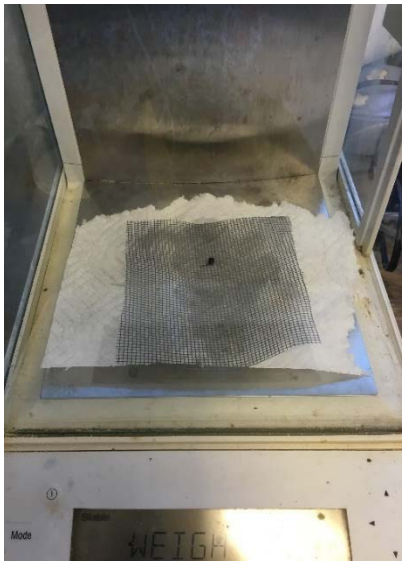

Figure 2: Apparatus for measuring tadpole weight (Browne, 2014).

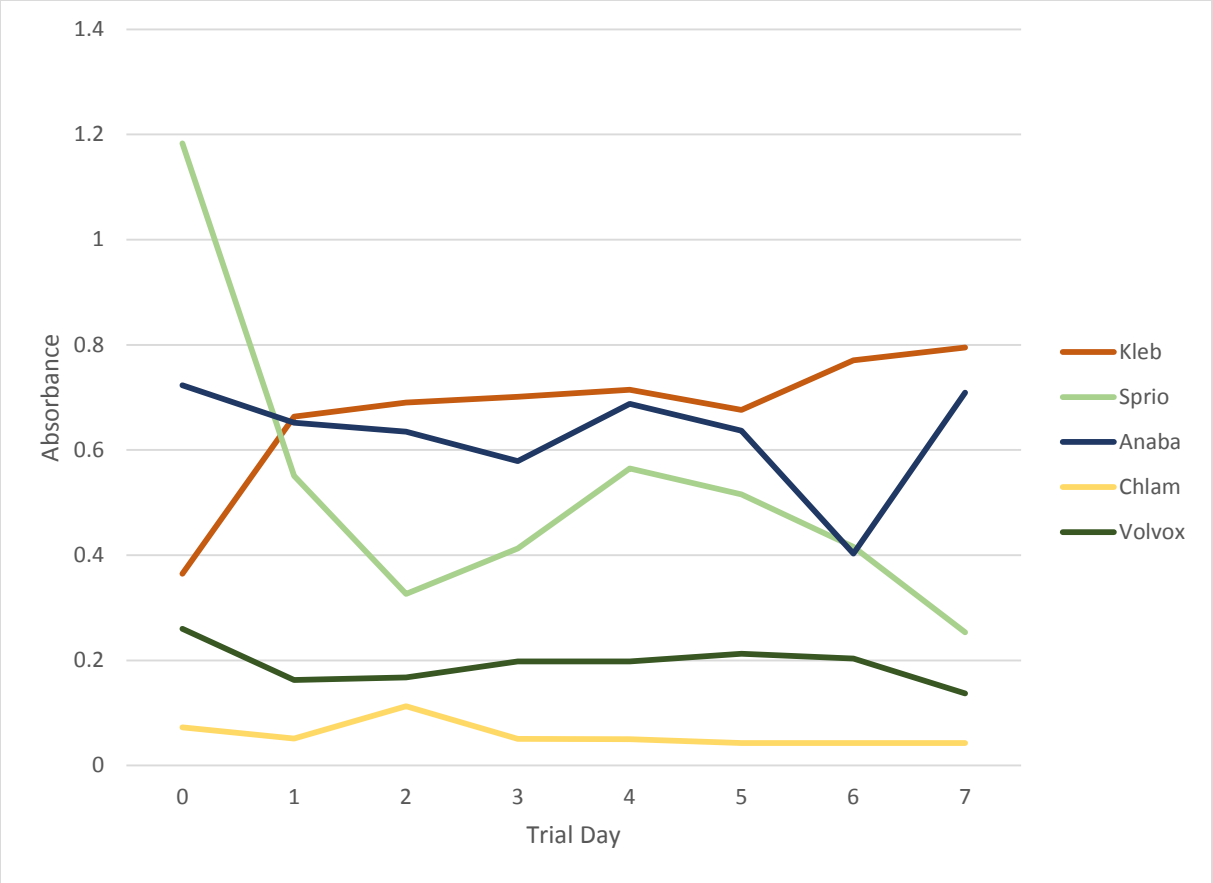

Figure 3: Growth rates of five algae samples over a week trial period to be used in a tadpole consumption study. Results of an ANCOVA reveal a significant difference in the interaction between day and algae type, yielding values: DF: 4, F: 3.7501, and P-value: 0.0137. Further linear regressions showed three classes of growth rates; positive, negative, and approximately zero, based on slopes with $R^2 > 0.1231$ and $p<0.394$.

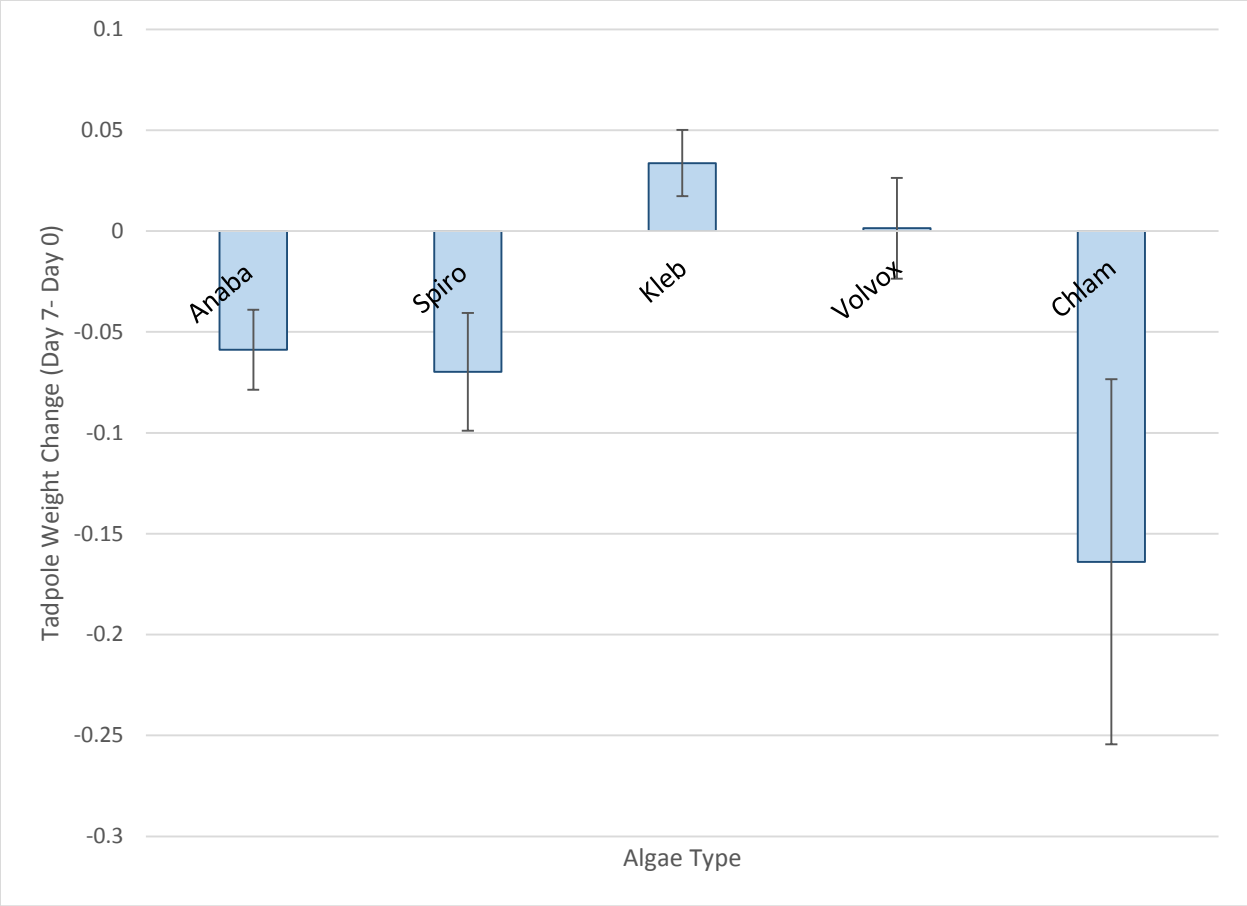

Figure 4: Average tadpole weight change between Day 0 and Day 7 of a week-long trial while being fed five unique species of algae. Results of an ANCOVA revealed no significant difference between algae type treatments on the change in weight of tadpoles over a week-long trial period. The ANCOVA calculated values of $F_{4,9}$: 2.0235 and p-value: 0.17455 and showed a trend towards significance.