Effects of Mediterranean dry year conditions on the survival of trematode-infected snails

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ABSTRACT

Worldwide populations of amphibians are declining. Some North American amphibian populations are under increased threat from limb malformations induced by the trematode *Ribeiroia ondatrae,* which maybe exacerbated by climate change. I studied the effects of age, and infection with *Ribeiroia* on the survivorship of the aquatic snail *Planorbella tenuis* in drought and normal moisture conditions consistent with seasonal pond in a Mediterranean climate. Moisture treatments had very little influence on survival ($p=0.2$). Age ($p=0.8$) did not affect survivorship. Infection had the greatest impact on a snail's ability to survive desiccation ($p<0.001$). The added of effects of *Ribeiroia* infection and moisture level were non significant in influencing survivorship ($p=0.4$). Age and moisture level were also non-significant factors ($df= 1$ $p = 0.6$) in determining survivorship. These results suggest that pond desiccation is ineffective in reducing snail populations. Infection by an undesired ectoparasite *Chaestogaster sp.* may have altered results in this study by lowering survivorship of snails. Based on observations of the ectoparasite, I recommend exploration of this ectoparasite for controlling the snail population of *Ribeiroia* infected ponds.

KEYWORDS

Planorbella, desiccation, *Ribeiroia,* climate, *Chaestogaster*

INTRODUCTION

Amphibian populations worldwide have been declining over the past decade (Carey *et al.,* 2001). By the early 2000s researchers were investigating sources of amphibian mortality for fear that amphibians, particularly frogs, would go extinct in the coming decades as a result of increased habitat loss and the spread of *Batrachochytrium dendrobatidis* infections (Carey *et al.,* 2001; Young *et al.,* 2001; Galloy & Denoel, 2010; Adams *et al.,* 2010). In the mid 1990s amateur observers noted an increase of malformations in amphibians which was linked to the declines (Souder 2000). Many scientists attribute the increase in malformations to direct anthropogenic causes such as the ozone depleting effects of UV-B radiation (Blaustein *et al.,* 1998), to pesticide runoff (Ouellet *et al.,* 2007), and to eutrophication (Johnson *et al.,* 2007). Field experiments by Johnson and others provided evidence of the source of these malformations: parasitism (Johnson *et al.,* 1999, Johnson *et al.,* 2002).

The main culprit behind limb malformations in North America is a trematode *Ribeiroia ondatrae* (Johnson *et al.,* 2002; Blaustein and Johnson, 2003). *Ribeiroia* uses snails in the family Planorbidae (Johnson *et al.,* 2004) to launch its attack on amphibians (Esch *et al.,* 2002; Esch & Fernandez, 1994). The parasite uses the snail to enter an asexual stage called the rediae which produces hundreds of copies of a swimming stage called cercariae that hunts down amphibian larvae and burrow into the hind limb region (Johnson *et al.,* 2004). This action disrupts development in the amphibian resulting in limb malformations (Sessions & Ruth, 1990). Eutrophication of waterways from agricultural waterways has indirectly aided the parasite by increasing the population of snails through algal blooms (Johnson & Chase, 2004, Johnson *et al.* 2007). Algal blooms, specifically, periphyton, serve as the main source of food for snails.

Besides eutrophication, another important anthropogenic factor that may influence parasite malformation in amphibians is climate change (Johnson & McKenzie, 2009). Climate change is expected to negatively affect aquatic systems in places with a Mediterranean climate such as California (Gastith & Resh 1999) which are characterized by cool wet winters and hot dry summers. It is expected that California will be subject to frequent and longer droughts (California Dept. of Water Resources 2009). One species of interest in this context is that of the snail *Planorbella tenuis* which that aestivates during

the high heat of summer and acts as host to *Ribeiroia ondatrae* (Johnson *et al.*, 2002). It is not clear how increasing the period of drought will affect the survivorship of these snails, which have been observed to carry over infection past desiccation (Lunde, unpublished data). After a drought in 2007 the snail population at a site with high levels of *Riberoia* infection was associated with a substantive decline in the snail population. It seems probable that snail survivorship over the summer period was lower than normal because of dry soil moisture and longer dry conditions (Lunde, unpublished data).

To study the effects of desiccation and infection with *Ribeiroia ondatrae* on the survivorship of *Planorbella tenuis*, I experimentally manipulated moisture levels in a lab setting to represent a range of conditions between a drought and a normal Mediterranean year in an ephemeral pond. I tested the follow hypotheses: a) snails in a drought year exhibit lower survivorship than snails in a wet year, infected snails in a drought year exhibit lower survivorship than uninfected snails, and old snails exhibit lower survivorship than young snails. The null hypothesis is that there will be no differences in all three cases.

METHODS

Site Description

Hog Lake is located at the UC Hopland Reserve in Northern California near the city of Hopland (39.032°N 123.079°W) . It is a seasonal lake that is a quarter of an acre in size. I chose this site because it had several years of moisture, snail population, and infection data available to me through Kevin B. Lunde. It was the source of the snails used for this experiment.

Study Species

Ribeiroia ondatrae

The Ribeiroia parasite has a complicated life cycle involving three different hosts. The cycle begins with the excretion of parasite eggs from the digestive system of the definitive avian host into a body of water. The eggs hatch in water and seek out the aquatic snails in the family Planorbidae (Dillon 2000). The parasite begins asexually reproducing in the snail gonads, absorbing the surrounding tissue (Esch et al., 2002), later releasing another swimming stage called cercariae that infects the frogs (Johnson et al.,

2004). If the snail dies before the cercariae are ready to be released, the parasite perishes as well. The cercariae will penetrate tadpoles in developing limb regions, shed tails, and burrow in the surrounding tissues (Esch & Fernandez, 1994). This action of burrowing in the hind limbs is the primary cause of amphibian limb malformations (Johnson et al., 2007). Limb malformations hinder movement and increase the amphibian host's vulnerability to predators (Johnson et al., 1999) which ensures that the parasite will complete its life cycle in the avian gut (Johnson et al., 2004).

Planorbella tenuis

Planorbella tenuis is a common pond snail found in seasonal ponds present at Hog Pond in Hopland, CA. The snails are hemaphroditic pulmonate snails in the family Planorbidae (Dillon 2000). The Mediterranean seasonal ponds are dependent upon the winter seasonal rains and a change in the weather patterns may adversely affect the timing of breeding and survival of the aquatic organisms in California (Beche & Resh, 2009). The P. tenuis snails are adapted for extended periods without moisture (Lunde pers. Observ; Dillon 2000). When water levels drop, the snails burrow from several inches to a foot into the soft muddy bottom of the pond. Previous research at this site has found that infected snails survive the summer aestivation period from late June or early July, reemerging at the start of the winter wet season in late November to early December when water has collected in the pond (Lunde unpublished data).

Experimental Design

I used a laboratory experiment to determine how trematode infection affects snail survivorship under drought conditions. The experiment was conducted in a greenhouse insectary at the University of California, Berkeley Oxford Tract in Berkeley, CA. In ten general purpose aquaria, I simulated the drying rates of Hog Lake, in a normal and drought year. Five tanks were assigned the "Normal" treatment and five were given the "Drought" treatment. Each tank had the following combination of snails: 5 old infected, 19 old uninfected, 19 young infected, 19 young uninfected for a grand total of 620 snails (620 = 62 snails/tank x 10 tanks). All the snails used in the experiment originated from Hog Lake or were the progeny of snails from the Lake. This site was chosen because of its proximity to UC Berkeley and the availability of data from past four years. The preparation for the study began in March 2009 while the experiment ran from September

20, 2009 to March 8, 2010.

To simulate the drying rates of Hog Lake in a drought and normal year, I placed 1 gallon of dechlorinated water into the Drought tanks and 2 gallons into the Normal tanks. All 10 gallon volume tanks contained a soil layer up to 12.5 cm taken from Hog Lake. Drought tanks were allowed to dry from the start of the experiment on September 20, 2009. Normal tanks were maintained at 40 cm of water until October 26, 2009 when the Drought tanks went dry. On October 27, 2009, I began adding dechlorinated water to the Normal tanks less frequently so that they could begin drying. The moisture levels were monitored with an Em 50 and Em-5 Environmental Logging System from Spectrum Technologies with EC-5 and EC-10 moisture probes inserted approximately 12 cm into the soil. Soil moisture data collected from Hog Lake served as a guide for appropriate drying rates in the experimental tanks (Fig 1).The Drought tanks were modeled after Hog Lake in 2008, which was a normal year (Lunde, unpublished data). The Normal tank drying rates were modeled after Hog Lake in 2009, which was a dry year (Lunde, unpublished data).

Snail Collection and Screening

Snails were collected from Hog Lake between Dec 2008 and May 2009. For this experiment, these snails are designated old snails. The old snails were used to rear the young snails (those snails born in the lab between May and July 2009) used in the experiment. I screened *Planorbella tenuis* snails from Hog Pond for any preexisting infections to avoid contamination with a different and undesired parasite, *Echinostoma* spp. Snails were placed in polypropylene tubes with 100 ml of distilled spring water and I looked for cercariae that were shed in the afternoon which is consistent with *Echinostoma* spp. behavior.

The snails were reared in a greenhouse in standard fish aquaria from March 2009 through September 2009 in order to acclimate them the temperature and humidity regime within the greenhouse. The snails were periodically fed boiled organic green lettuce *ad libitum* to avoid the toxic contaminants from petrochemical agriculture.

Parasite Egg Collection and Infection

Snails were infected with parasite eggs collected from the feces of laboratory rats that were fed isolated metacercariae from infected intermediate amphibian hosts in the Pieter

T.J. Johnson laboratory at University of Colorado at Boulder. The excrement samples were sent in weekly or biweekly batches from March 2009 through July 2009. Once parasite eggs were separated from the fecal samples, they were placed in a gallon of dechlorinated water in an incubator for 3 weeks to mature to their first miracidia stage where they could be used to infect the snails (Johnson *et al.,* 2004). The incubator was maintained at a temperature at or above 28 ℃, the optimal temperature for development (Lunde pers observ), kept well oxygenated with aerators, and kept dark to prevent premature hatching. Every week I refiltered the batches to get rid of bacteria that breakdown *Ribeiroia* eggs. I took two drops of the batch to examine the egg density and development; It was important that I found the majority of eggs in stage 3, immediately before hatching, so that I could apply them to the snails.

Snail Infections

To maximize the chance of infection, I placed the snails in a separate aquarium in 1-2 inches of water with the filtered rat excrement and exposed to full sunlight. After seven days the snails were removed, placed in a different tank and maintained until the start of the experiment. I did this from May 2009 through July 2009. 268 total infected snails were produced from this process.

All the uninfected snails collected from Hog Lake were kept in a large tank (3x1x1 ft^3) with a half inch layer of crushed coral to provide a source of $CaCO_3$ for shell formation and maintenance. Snails that went through an infection session were kept and maintained in a different set of tanks (16x 12 x 10 in) also with crushed coral.

Infection levels were checked by shedding the snails after ten weeks, the time required for an infection to mature (Johnson *et al.,* 2004). Shedding is the release of cercariae from the snail, and can be observed with the naked eye. The cercariae are released from the asexual rediae stage of the parasite which seems to continue to producing cercariae for the duration of the snail's lifetime as seen from field observations of infected snails emerging following the winter rains. Two batches that had too few infected snails (infection proportion <30%) were re-infected and yielded nine additional infected snails out of an initial forty, the rest were used for the uninfected group.

Tagging Procedures

In late August and early September 2009 all snails were individually given a

combination of two tags designating their age and presence of infection. Individual markers were used to monitor survivorship within the appropriate age and infection treatment. The "Bee" tags were attached using Fast Action SuperGlue from a local hardware store. The snail shell was dried with a Q-tip, using a fine tip paintbrush a small drop of the glue was applied to the whorl, the marker was applied with a fine tip tweezers from a dissection kit, and pressure applied for 15-45 seconds.

Data Collection

After each tank dried, late October for the Drought treatment and late November for the Normal treatment, I removed snails that had not burrowed into the mud and were dead. I sized these snails and counted them to look at any trends in the pre-desiccation period. In March, all the tanks were refilled with dechlorinated water, seven months after the initiation of the study. The Drought tanks were dry for six months while the Normal tanks were dry for five months. Each surviving snail was recorded.

Data Analysis

I calculated percent survivorship to use for statistical testing because of the unequal sample size from having too few year one infected snails. I used Welsh t-test to conduct significance tests on age, moisture, infection. Welsh t-tests were also used on age and infection to compare across Drought and Normal treatment. I used ANOVA to detect any interactions between Age, infection, and moisture treatments using R. I used Binomial Confidence Intervals to do a qualitative Chi square tests; if the 95% confidence interval means overlapped then the difference was not significant. All percent survivorship data were arcsin transformed to turn them into continuous variables in order to fulfill the requirements of ANOVA and t-tests.

RESULTS

Moisture

Desiccation lasted for five months in the Normal treatment and six months in the Drought treatment. Drought tanks were maintained near a 15% soil moisture following desiccation and Normal tanks maintained near a 25% moisture level (Fig. 1). The percent difference between moisture levels is the experimental variable between Drought and Normal tanks which was 10 % difference in soil moisture. Field soil moistures are

provided as a comparison (Fig. 2).

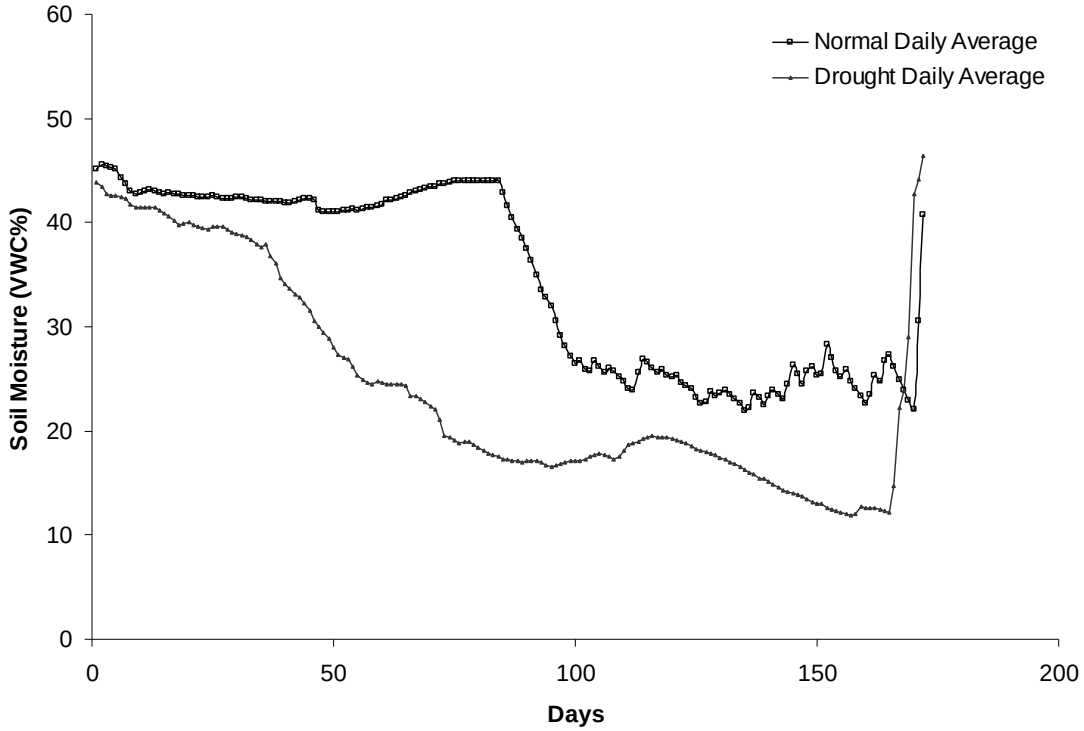

Figure 1. Percent soil moisture of the drought and normal treatment tanks. The gentle drop in soil moisture mimic natural trends in pond desiccation at Hog Lake.

I allowed the drop in percent soil moisture for the Drought tank to begin immediately. The normal tanks remained wet for 60 days, but the drop in percent soil moisture began around 80 days after the start of the experiment. The staggered end of the normal soil moisture is a result of attempts to simulate the start of the rainy season as seen in Hog 2009 data (Fig. 2).

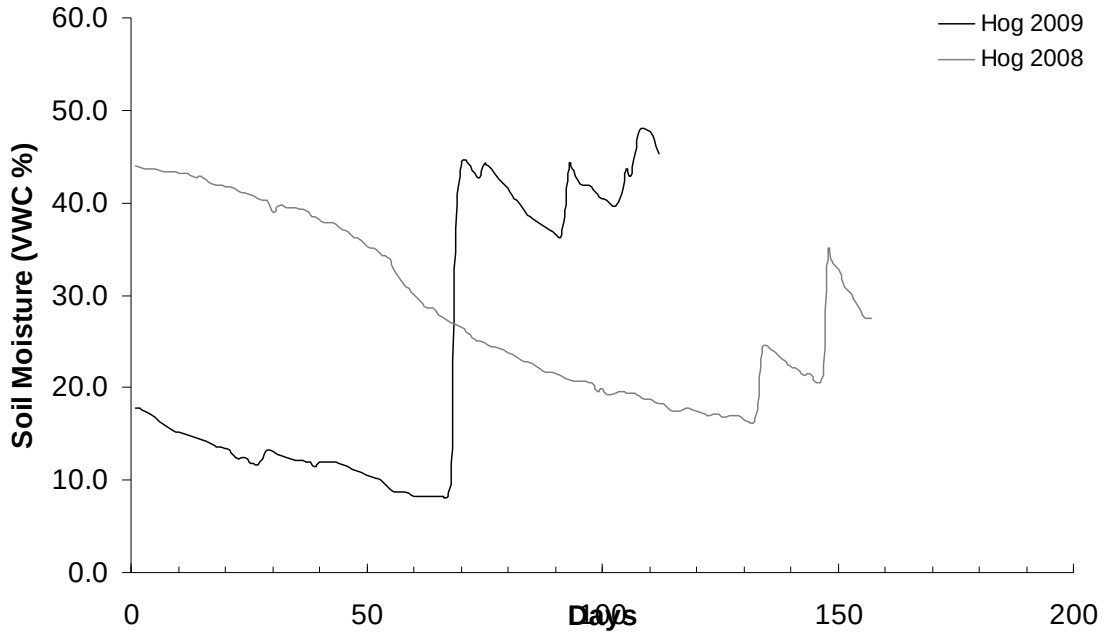

Figure 2: **Percent soil moisture of Hog Lake during normal year 2009 and drought year 2008.** This data was used as a guide for the drying rates in the laboratory study. Drought year measurements were taken with a moisture meter placed 5cm deeper into the pond substrate.

Snails were collected over two weeks following rehydration. Survivorship was scaled as proportion of those surviving the whole study out of those that survived up until desiccation because of the unequal sample size of the year one age snails. Snails showed no significant differences in survivorship between Drought and Normal treatment ($df = 1$, $P = 0.10$). Percent survivorship was slightly higher in the drought treatments (Fig. 3), but this relationship was not statistically significant.

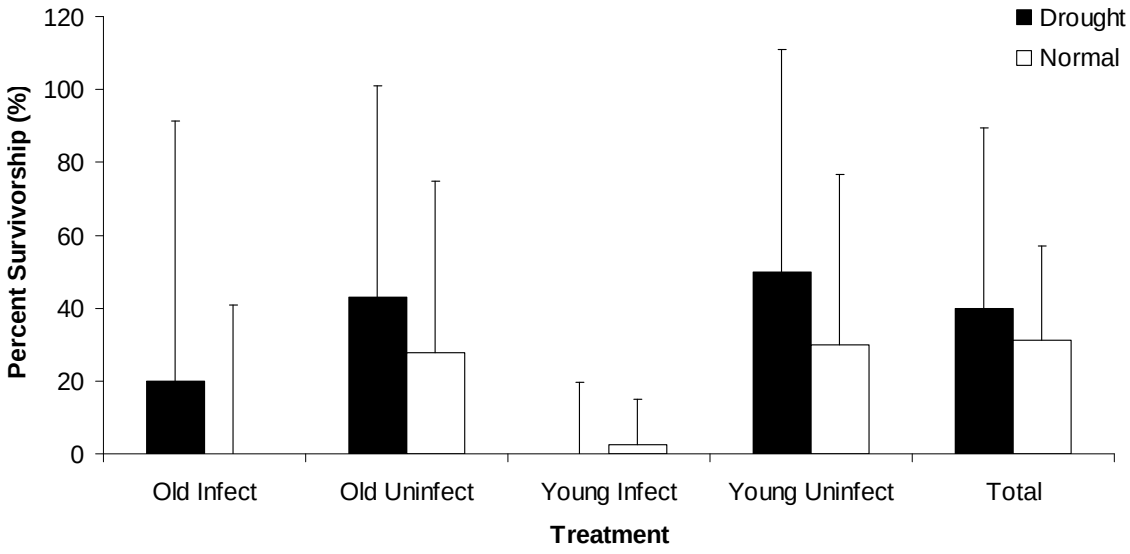

Figure 3. **Effect of drought on the survivorship of *Planorbella tenuis* among different groups** . Comparison of "Total: is the sum of all groups is between Drought and Normal tank treatments after six months (Drought) and five months (Normal). 95% Upper confidence limits included.

Infection

Uninfected snails had higher survivorship over their infected cohort (Fig. 3) which was statistically significant (t = 0.1714, df = 38, p-value = 0.8648). This significance is apparent when comparing infection status within each treatment as seen by the lack of the overlap of mean survivorship in the 95% confidence intervals (Fig. 4). A Welsh t-test of arcsin transformed percent survivorship shows that the higher survivorship among uninfected snails is greater and more significant in the drought treatment (t = -3.5669, df = 11.039, P <0.01) than in the normal treatment (t = -3.2687, df = 10.036, P < 0.01).

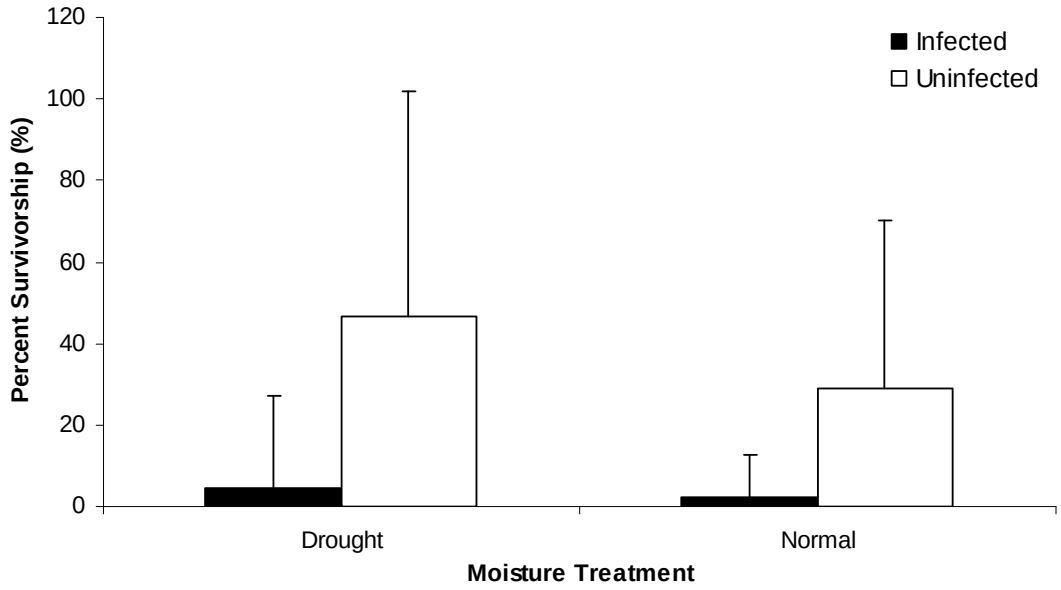

Figure 4. **Effect of Ribeiroia infection on survivorship of *P. tenuis***. Comparing *Ribeiroia* infection status within drought and normal treatments. 95% Confidence Intervals are shown.

Age

An assessment of age influence on survivorship revealed no significant difference between drought and normal treatments ($t = 0.1714$, $df = 38$, $P = 0.8648$). The lack of significance is apparent when looking at percent survivorship within each moisture treatment, for example the year one and year zero snails had the same survivorship within the drought treatment (Fig. 5).

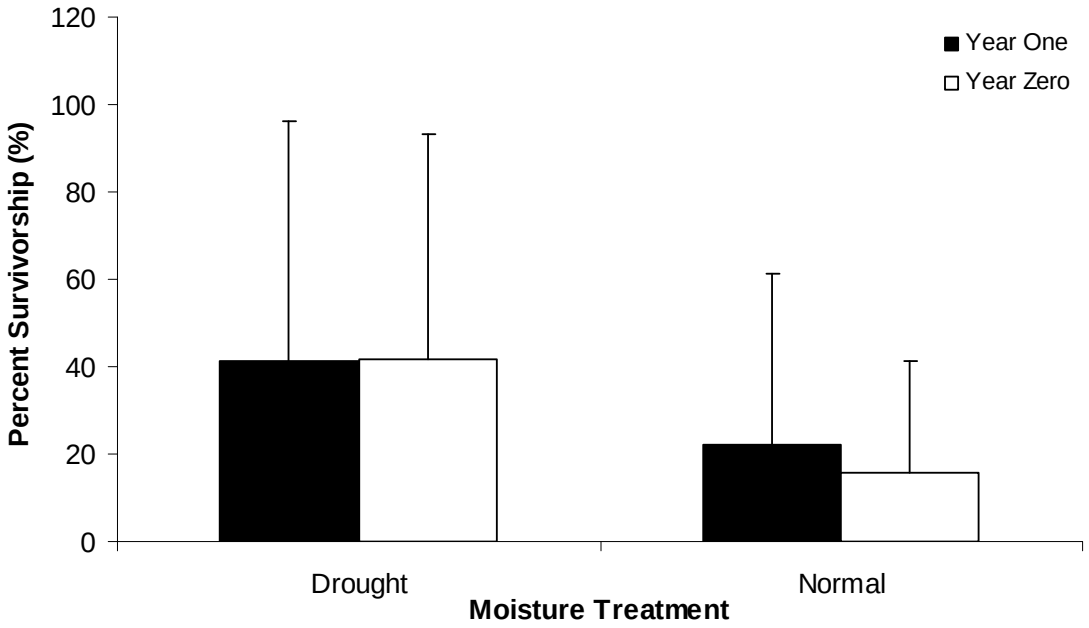

Figure 5. **Effect of age on survivorship of *P. tenuis***. After desiccation percent survivorship of *P. tenuis* comparing year one and year zero survivorship within drought and normal treatments

Interactions

I analyzed two way and three way interactions using ANOVA. I used an Arcsin transformation of percent survivorship to transform the data into continuous variables with normal distribution. The age and moisture interaction was not significant (df= 1, P= 0.6). Likewise, age and Infection interaction was not significant (df = 1, P= 0.4). Infection and Moisture interaction was also not significant (df =1, P= 0.6). An examination at the three way interaction between the variables showed not significance as well (df= 1, P= 0.4).

DISCUSSION

I began this experiment with the assumption that increased age, *Ribeiroia* infection, low moisture levels are factors that contribute to lower survivorship in *Planorbella tenuis.* I expected the data to follow certain patterns: infected snails would exhibit lower survivorship than non-infected snails, and snails in the Normal moisture treatment to exhibit higher survivorship than snails in Drought treatment, and that year one snails exhibit lower survivorship than year zero snails.

Moisture

Contrary to my prediction, soil moisture had no noticeable effect on survivorship. The effect of drought was not apparent when looking at interactions with age (Fig. 5) or

infection (Fig. 4) refuting my hypothesis that snails in the Normal treatment would have shown higher survivorship. This result contrasts with a previous laboratory study which found that desiccation did have an effect on survivorship and snails in their hydrated treatments had significantly higher survivorship than snails in their aestivated treatment (Sandland & Minchella, 2004). A parallel field study conducted over five months also found that survivorship during desiccation dropped precipitously to between 0.5% and 1.7% survivorship (Sandland and Minchella, 2004). An important difference between Sandland and Minchella's 2004 laboratory study and my own methods is that the desiccation period was only two weeks as compared to five and six months in my study. My results do follow the trends of their field study with a severe drop in the survivorship of infected snails. However, I cannot say that my lab results corroborate their field results because field studies are subject to various uncontrolled variables. I believe that drought is a stressor on snails. Aestivation is energy intensive and takes a toll on snails because of the loss of fluid and the energy required for maintaining stasis (Arad, 2001).

Infection Presence

As expected, infected snails showed lower survivorship than uninfected snails. This result differs from a similar study of *Lymnaea elodes* which found that infection by trematode was not a very significant factor (Sandland & Minchella, 2004). However, the parasite of *L.elodes* used in that study (*Echinostoma* spp) is not one that causes significant mortality in snails as compared to *Ribeiroia* (Sorenson & Minchella, 2001) because *Echinostoma* has a different life history than *Ribeiroia* (Esch *et al.,* 1991). In addition *Lymnaea elodes* is adapted to unpredictable seasonal pond conditions (Brown, 1985) unlike *P. tenuis* which is adapted for predictable seasonal pond conditions.

My results were also contrary of a field study involving infected and uninfected snail survivorship that found no difference in survivorship between infected and uninfected snails (Negovetich & Esch, 2008). A laboratory study by Keas & Esch (1997), on a related question found no differences as well. The subject used for both studies was, *Helisoma anceps*, a pulmonate snail which is a relative of *P. tenuis*, and the parasite *Haligupus occidentalis*, a trematode that also castrates its host. The difference between my results and their studies is that their study occurred in North Carolina under conditions where the pond was always wet. Field studies tend to have many confounding

factors such as differing food levels and interactions with other coinhabitants (Negovetich & Esch, 2008) Therefore the effect of parasitism may have not been detectable. Also, maybe *H. anceps* is more resilient to trematode infection than *P. tenuis*.

Age

My predictions for age were based of the r-selected life history patterns exhibited by *P. tenuis* in Hog Lake; a large percentage of the year one snails senesce during the late spring and early summer after reproducing (Baker & Cleave, 1945). Contrary to my predictions age was not a significant factor in survivorship after desiccation. When examining age in the uninfected group survivorship trends were similar between the Normal and the Drought tanks. My results are in line with studies where age was an unreliable factor for indicating survivorship or infection prevalence (Negovetich & Esch, 2007). However the studies mentioned were conducted in the field and not in the laboratory, so extrapolation may be premature.

Interactions

My study had an underlying assumption that age, infection, and moisture are interacting variables. The results revealed that this assumption was incorrect. An interaction between infection and moisture was assumed to have an additive effect on the snails but this was not observed. Although parasitic infections and drought are an immediate physiological impediment to a snail's survival (Sorensen& Minchella, 2001; Sandland & Minchella, 2004) the survivorship was the same in both tank treatments (Fig 4). Considering the snails' adaptation to dry conditions it may have been a mistake to assume moisture would interact with infection to decrease survivorship. The lack of dependence between age and infection was surprising. The influence of age on the severity of parasitic infection has been posited in the literature (Sandland & Minchella, 2003; Fernandez & Esch, 1991) and tested but in relation to the question of gigantism, not survivorship. It may have been a poor study design to look for an interaction between age and moisture considering that the tanks differed in their moisture levels by 10% and that a one year difference between snails was not enough. I was surprised to find that there were no interactions between all three factors. It may have been because of the vast mortality experienced by the infected snails and the additional infections by an aquatic oligochaete. There were too few infected snails and not enough uninfected snails to truly

show the effects of moisture and age.

In summary, moisture levels had no detectable influence on survival. Infection had the greatest impact on a snail's ability to survive whether in the Drought or Normal treatment and age was not an important factor affecting survivorship. The most important implication is that there are no additive effects of infection and moisture on survivorship.

Limitations

Some of the problems with this study preclude me from making inferences to other systems. Unequal sample sizes were a problem because I did not have enough old infected snails. This made the acceptance of statistical analyses difficult, in addition to skewing the data. The reason for the low number of old infected snails was the stress of infection and the oligochaetes which first appeared in the preparation tank for that group.

The observation of higher survivorship in the drought tanks can by explained by infection by the oligochaete *Chaestogaster* sp., an ectoparasite that attaches to the snail (Patzig & Schmid, 1981) and is thought to be commensal. The Normal tanks which were wet for a month longer than the Drought tanks had sufficient time to accumulate the infection, which appeared to grow worse until the dry down. Also the steep drop in soil moisture in the Normal tanks may have had a negative impact as well (Fig 1).

Maintaining soil moisture in the Normal tanks was a challenge because it would dry too quickly as compared to the Drought tanks which would dry too slowly. This would result in fungus growth in the Normal tanks so I refrained from adding water until the fungus disappeared. Caution is suggested when extrapolating results to other systems particularly those with different snail species. The results here should be only used to infer on processes in seasonal Northern California ponds involving *P .tenuis*.

Conclusion

Based on the limitiations of this experiment, I recommend further studies into the effect of desiccation on snail survival. The Drought treatment should be at least two or three months longer than the Normal treatment; beyond what *P. tenuis* normally faces in the field. This exacerbation of conditions may reveal potential effects of future climate change on snail survivorship.

The knowledge yielded from this study can be used within the larger context of amphibian malformations. Amphibians in North America are experiencing loss of habitat

(Carey *et al.*, 2001; Young *et al.*, 2001) which forces them to concentrate around artificial habitats such as farm ponds for cattle. These pond locations are strongly correlated with malformation hotspots (Johnson & Sutherland, 2003). I initially believed that if my hypotheses were correct in that extended desiccation caused lower survivorship in snails, then drying down these ponds for an extended period of time could be used in a manner to regulate the snail populations. However, desiccation doesn't appear to be a viable management strategy at this time. Infected snails will die off regardless of moisture level thus the idea that drying out ponds as a means of controlling the spread of *Ribeiroia* infection among amphibians is not ideal. *Ribeiroia* will be a threat to amphibians as long as there are active and healthy snails during time where amphibian larvae are active and growing (Johnson *et al*., 1999, 2002). Any means of controlling the parasite must be timed for when the vulnerability of amphibian larvae are at its highest.

With the obtained results and the possible negative effect of *Chaestogaster* on snail survivorship, I recommend research into the use of these ectoparasites (Patzig & Schmid, 1981) a possible biological control agent stress snails. Also, *Chaestogaster* has been observed as a symbiont on some snails protecting them from infection by ingesting the trematode (Michelson, 1964; Ibrahim, 2006). Biological control would preclude the drying out of ponds which may be financially unfeasible and unreasonable for farmers and ranchers who need these ponds to water their crops and livestock during the high heat of the inland California summers. Considering extinction pressures of habitat loss and *Batrachochytrium dendrobatidis* infections on amphibians it is important that we reduce the additional pressure of parasitic trematode by finding an economically viable method of controlling the aquatic snail hosts in North America.

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