Nematode Burdens in *Rana muscosa*

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Abstract: Disease has been implicated as a major concern in population declines of the mountain yellow legged frog, *Rana muscosa*. The knowledge regarding parameters that increase susceptibility to infection and death by the fungal pathogen chytridiomycosis is understudied, yet desired to help implement effective conservation methods. This research presents an analytical study that correlates parasitic nematode burdens within the digestive tract to infection of frogs from three Sierra Mountain localities, Milestone basin, Sixty Lakes Basin and Cow Creek. A sample size of 121 frogs spanning the aforementioned sites was utilized. Dissection of the gastrointestinal (GI) anatomy of *R. muscosa* was used to quantitatively measure nematode densities at three locations, the stomach, duodenum and lower GI tract. The results show a significant difference between higher mean burdens at an infected site (Milestone Basin) and lower nematode means at an uninfected site (Sixty Lake Basin) as reflected by the non-parametric Wilcoxon rank sums and Kruskal-Wallis statistical tests. Higher densities of nematodes were also observed in the duodenum relative to the stomach and lower GI tract. All three locations were determined to be significantly different. Non-significance was observed with mean comparisons of seasonality, sex, and most lakes within each study site. Identification of a nematode species has signaled a new species of genus *Aplectana* within host frogs at Sixty Lake Basin. Thus, a relationship exists regarding numbers of nematodes and diseased specimens of *R. muscosa.*

Introduction

The decline of global amphibian populations has become of major concern to ecologists throughout the world (Blaustein & Wake 1995). From introduced exotic species (Knapp & Matthews 2000) and loss of natural habitat (Fisher & Shaffer 1996) to predation by fish (Vredenburg 2004), the species abundance of many frog populations has fallen to dangerously depleted levels. In particular, *Rana muscosa*, located in the California Sierra Nevada Mountains, has been declining at an alarming rate over the past two decades. According to Grinnell and Storer (1924), *R. muscosa*, the mountain yellow legged frog, was once one of the most abundant species of vertebrates in the Sierra localities, but has recently fallen to endangered levels. It is estimated that as much as a 95% reduction in population has characterized this decline within the past century (Berger et al. 1998). The setting for many of these declines has occurred within mostly pristine habitats, which has caused great concern among herpetologists and conservationists around the world.

Frog and amphibian species play essential roles in ecosystems and have been identified as effective biological indicators of environmental health (Duellman and Trueb 1986). Causes that may have contributed to this remarkable drop in species abundance include the introduction of exotic trout into native territories, introduced predator frog species, fragmentation of natural habitat, and of recent concern, the fungal pathogen *Batrachochytrium dendrobatidis,* which is the cause for the fatal disease, chytridiomycosis (chytrid) (Berger et al. 1998). Currently, it is unknown whether this disease is endemic or novel in origin, although it is undoubtedly instigating declining amphibian populations throughout the world (Retallic et al. 2004). The mystery surrounding chytrid is a question that has become the focus of recent research and work in lab and field studies.

The chytrid fungus' mechanism of action moves though the keratinized skin cells of adult frogs. Mouthparts of tadpoles are high in the protein and may be infected, although only post-metamorphic frogs are susceptible to death (Longcore et al. 1999). Due to the relatively recent documentation of this disease, a limited understanding of how and why amphibians are affected by chytrid exists. Characteristics that increase susceptibility for infection are mostly unknown.

The presence of nematode parasites located in the gastrointestinal tract of *R. muscosa* is a variable that has not been well studied or analyzed. While it is unspecified what role and impacts

these invertebrates may play in the life history of the mountain yellow legged frog, the presence of such organisms may be viewed as an indication of decreased health. As shown in previous studies related to the common laboratory mouse, *Mus musculus*, growth, immune system functioning, diet intake and general survival can be greatly affected due to the presence of parasites (Kyriazakis et al. 1996). Common vertebrates, such as lab mice that were infected with intestinal nematode parasites, *Heligmosomoides polygyrus,* often displayed a modification in physiology. Increased organ size, increased metabolic rate, and alterations in body mass have been described. Alterations in body resources often led to decreased life expectancy, and/or lower fitness levels in the study species. (Kristan and Hammond 2000, 2001). Within studies regarding amphibian species, such as the northern leopard frog, *Rana pipiens*, parasite infection was cited as a cause for sudden death in normally healthy populations (Hsu et al. 2004). Limb deformities were also correlated to parasite infection within the species *Xenopus laevis* in California (Loeffler et al. 2001). Increasing the knowledge and understanding of parasite presence will be vital to understanding the appropriate actions that will be needed to implement effective conservation efforts to the declining population levels of *R. muscosa*.

It will be difficult to determine a causation effect between parasite burden and chytrid infection, but this study only aims to help define what relationship may exist between a parasitic worm and *R. muscosa*. An examination of nematode densities will be accomplished through dissection and identification of a frog's corresponding physiology. The impetus of this research is to investigate the hypothesis that nematode presence is higher within diseased specimens. The antagonistic reputation of nematodes is the basis for this belief. A testing of this hypothesis will add to the understanding of the effects and vulnerability of *R. muscosa* to infection.

Methods

To determine if any correlation exists between this disease and parasite burden, field specimens of *R. muscosa* were analyzed. Specimens in this sample were collected during the summer months of 2004 at three separate locations, Cow Creek, Sixty Lake basin in King's Canyon National Park and Milestone basin situated at the heart of Sequoia National Park. Juvenile and adult specimens were collected by Vance Vredenburg, John Parker and Tate Tunstall from the department of integrative biology at U.C. Berkeley. The Collection periods spanned from June to September of 2004.
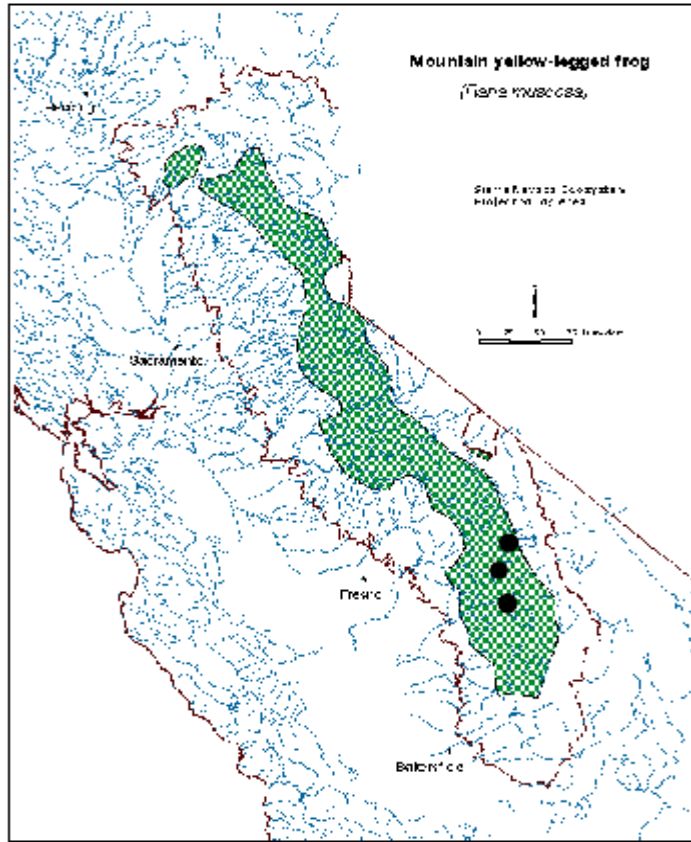
Figure 1. Distribution and study sites for *R. muscosa*. The green shading represents historical localities. Cow Creek, Sixty Lake and Milestone Basin are represented by black dots from north to south, respectively.

Upon visits to these three sites, a sample size of 121 specimens was amassed. Specific lakes within these localities differed on their infection level and were each sampled at early and late periods throughout the summer months. Thirteen lakes comprised the study sites, six from Sixty Lake Basin, six from Milestone Basin and one from Cow Creek. High diseased frogs originated from infected lakes, and healthy frogs were derived only from sites where chytrid was not detected. Lakes were characterized by an increase in infection during the late months, reflecting the higher proportion of diseased frogs within the sample size.

The specimens were euthanized in the field by an anesthetic overdose of MS-222 and then fixed in formalin-filled containers. Brain, liver and blood samples were drawn from each individual in the field. The specimens were then transported to the Museum of Vertebrate Zoology at UC Berkeley, where this study was conducted

Precautions were taken to ensure against personal bias. The majority of the methods used in this experiment were conducted blind. sample, and the identity of sex, infecti remained unknown throughout a major

To examine the presence of nemato required. Originating from the throat to running the ventral length of the body.

A disscted frog according to the experimental methods. A vertical incision is made from the stomach (shown) to the colon. Contents within the digestive tract are then removed and placed in vials.

structural mesentery tissue by a fine scalpel. Once free from the body cavity, the stomach's interior was exposed via dissecting scissors. Contents of the stomach were then removed and placed in a 15ml glass vial with 70% ethanol. The sample was then labeled with tape and marked with the individual's identification number and the region where the contents were removed. The remaining length of the GI tract was then opened, revealing the duodenum (small intestine) and lower GI. Contents within these regions were removed, transferred and labeled as previously described. Many specimens were previously dissected in the field and displayed an emptying of GI contents within their formalin containers. To resolve this issue, contents of the containers were collected with tweezers after being straining through coffee filters. The collected material was then stored in the corresponding vial where a notation of the collection differences were recorded.

After all specimens were dissected, a count of the nematode quantities was required. Contents from the three vials of each frog were individually examined under a dissecting scope. A probe was used to count the number of nematodes for each vial, and a total quantity was calculated per individual. Random samples were photographed at 40x magnification under a microscope.

An identification of nematodes was desired to the species level. Valerie McKenzie from the University of California, Santa Barbara became the main contact for this accomplishment. With these data, it will become possible to explore the possibilities of a correlation between parasite infection and disease.

Observation of the raw data, comparing Milestone, Sixty Lake and Cow Creek nematode burdens demonstrated a non-normal curve. A test of normality was performed using log transformed data, but also suggested a non-normal distribution. Therefore, non-parametric analysis by the Wilcoxon rank sums and Kruskal-Wallis test was required. A Tukey-Kramer test was used to distinguish significant means when appropriate. Due to the non-normality of the total sample size, it was assumed sub-samples within this population will also reflect a similar condition. Due to the non-normal distribution of parasite biology, the application of non-parametric statistical tests will be used consistently throughout the methods.

Results

86% of the observed specimens displayed at least one parasite present within the GI tract. The lowest nematode burden was 0, and the highest was 81. The majority of frogs exhibited a presence of nematodes in both the duodenum and lower GI regions, with the stomach displaying nematode presence with the lowest frequency. The mean number of nematodes within an individual was 13. Of the 138 species that were collected, 14 were not dissected due to difficulty in dissection techniques. Small size or desiccation were the most common reasons for omitting dissection
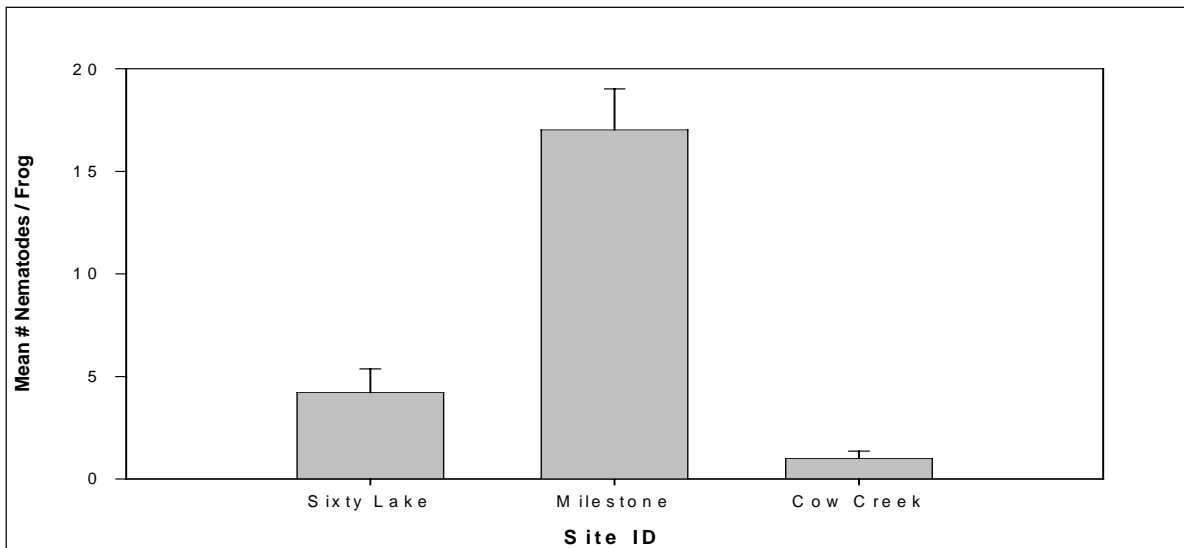
Figure 2. The mean distribution of nematodes between the 3 study sites. A non Parametric Kruskal-Wallis test with 95% confidence levels showed a p-value = .002. Sixty Lake (n = 28) and Milestone (n =

Figure 3. Distribution of nematodes within lakes at Milestone Basin. A non-parametric Kruskal-Wallis test with 95% confidence levels showed a p-value = .002. Lakes 20197 and 20198 were determined as significantly different by the Tukey Kramer comparison of means.
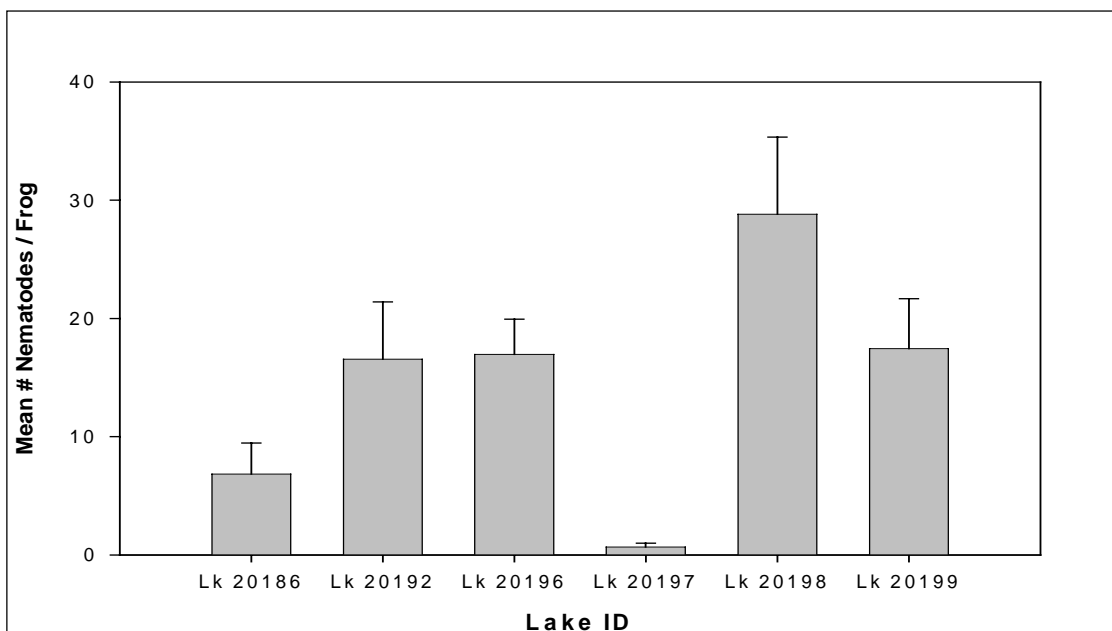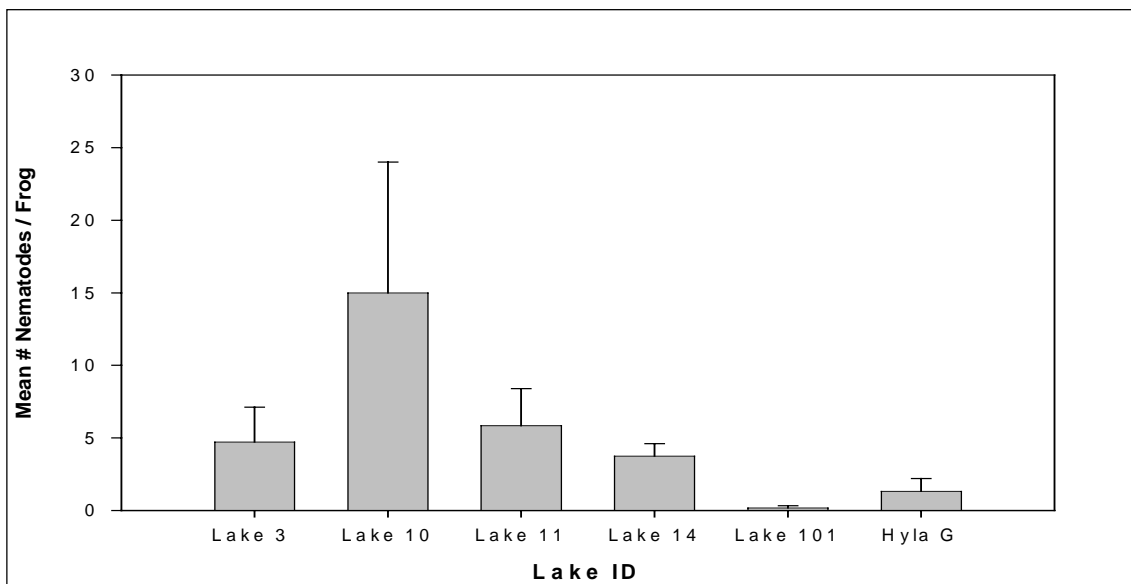

Figure 4. Distribution of nematodes with Sixty Lake Basin habitats. A non-parametric Kruskal-Wallis test with 95% confidence levels showed a p-value = .0115. A Tukey-Kramer comparison suggested a difference between lakes 10 and 101.
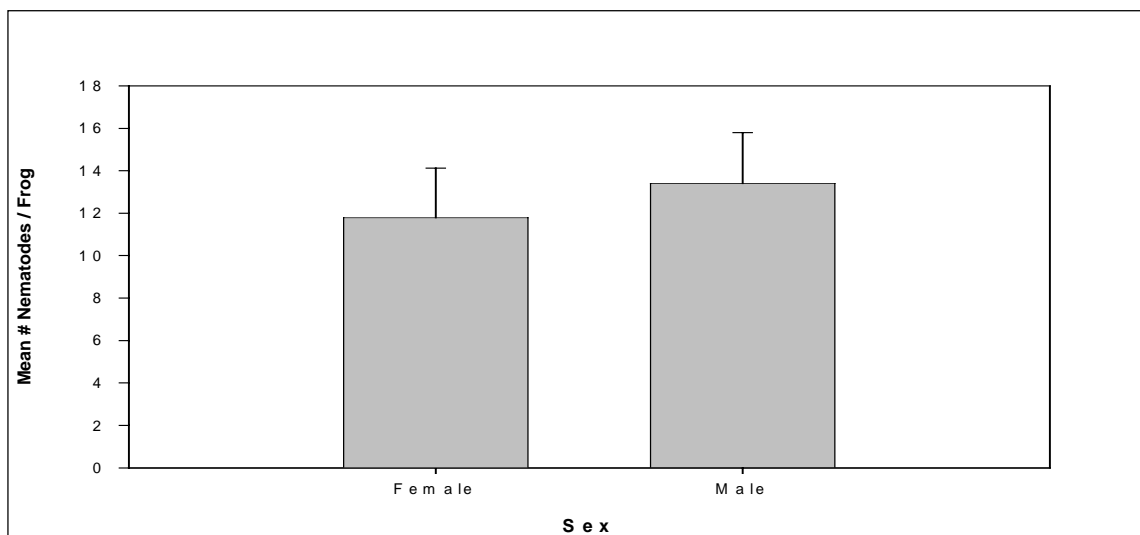
Figure 5. The mean distribution of nematodes between sex. A Wilcoxon rank-sum test with 95% confidence levels showed males (n = 53) and females (n = 45) as non-significant. P-value = .7824.

Figure 6. Seasonal differences in nematode distribution. June and July (n = 53) comprise the "early season". Aug. and Sept. (n = 58) comprise the "late season". A Wilcoxon rank sums test with 95% confidence levels calculated a p-value = .0698.

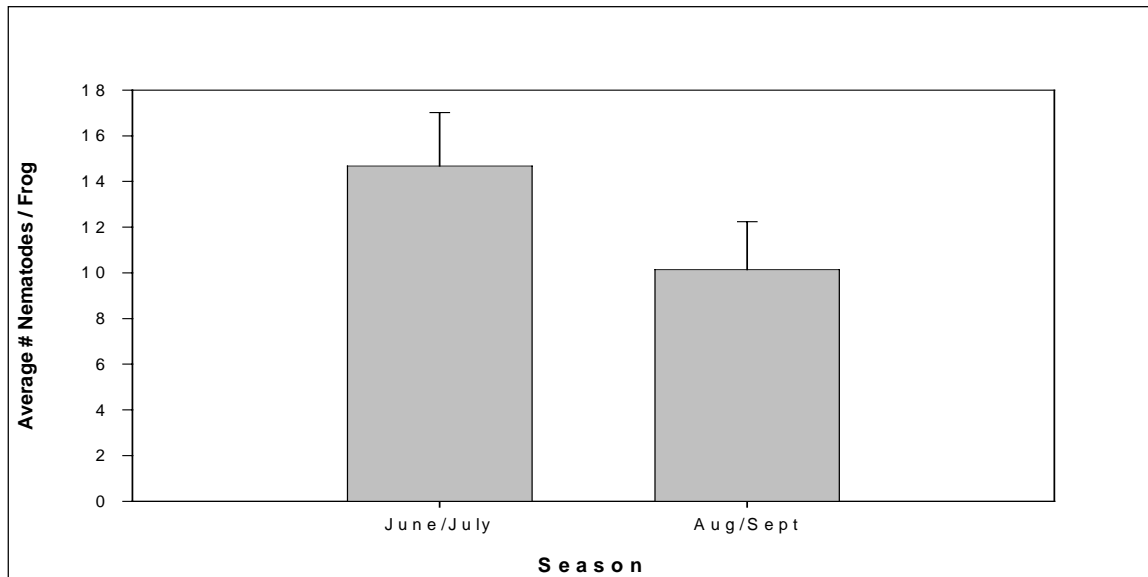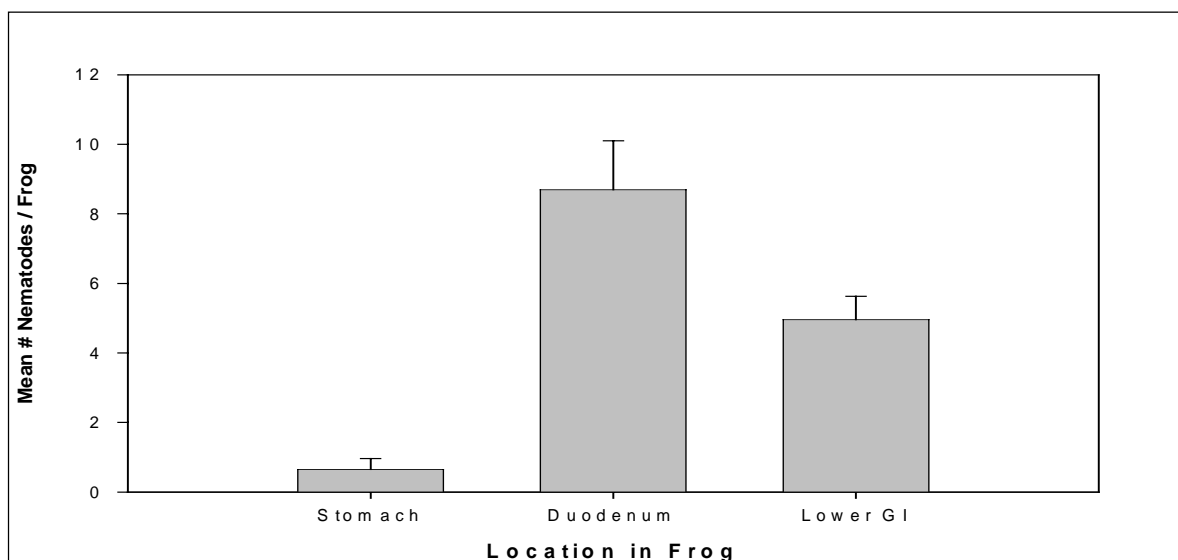Identification of one species of nematode was described as follows: order: Ascaridida,

Figure 7. Nematode distribution within the body of *R. musocosa*. The stomach (n = 110), duodenum (n = 110) and Lower GI (n = 110) comprise the upper, mid and lower sections of a frog's digestive tract. 95% confidence levels with a Kruskal-Wallis test show a highly significant value of <.0001. All three locations are significantly different according to the Tukey-Kramer test.

Figure 7. A male nematode of the genus *Aplectana*. This individual was discovered in the duodenum of a male frog within Sixty Lake Basin.

superfamily: Cosmocercoidea, family: Cosmocercidae, and genus: *Aplectana*. From this point, the literature does not allow further differentiation, likely signaling the identification of a new species. This species of nematode was from Sixty Lake Basin and was pulled from the duodenum of the frog. This is may not be the only nematode species present within the total sampled population. 1586 nematodes were collected from the 11 lakes and 121 frogs with only one worm from a frog in Sixty Lake Basin analyzed. At this point, 1585 worms still remained to be identified an no other identifications are currently in progress. It is uncertain how many different parasite populations may be present.

Discussion

The only site that was characterized as infected with disease was Milestone basin, even though most frogs within the sampled population displayed some level of nematode presence within one of the three observed anatomical regions. Sixty Lake displayed low levels of infection by an isolated incidence at lake 14 during the tail end of the season, but for the purposes of this study, is being treated as uninfected. The segregation and low infection status justify this. Specimens were collected at lake 14, but only 3 were observed within the methods of this research. With significantly different mean nematode burdens of 14 at Milestone and 4 at Sixty Lake, it may be reasonable to believe disease and

nematode densities generally have a positive correlation with one another. Infected frogs from Milestone generally displayed greater burdens than healthy specimens at Sixty Lake.

While Milestone and Sixty Lake were determined to be significantly different, an uninfected Cow Creek could not be statistically differentiated. Although this site is not statistically different, it is difficult to confidently compare the nematode means to Milestone and Sixty Lake. An issue with Cow Creek was its low sample size of 6. This reduced the power of the findings relative to the populations of Milestone and Sixty Lake, in which sample sizes of 87 and 29 were available. It is due to this decrease of power that Cow Creek cannot be easily compared to the other sites with the same confidence as would be desired. This issue may be resolved in future studies with a greater quantity and more even distribution of samples collected at the study sites.

Due to the significant difference between the main study sites of Milestone and Sixty Lake, an interest would be to examine what differences exist between the specific habitats within the basins. Milestone possessed two significantly different lakes, 20197 and 20198. Infection was first detected at the former site, with the latter exhibiting a late season disease presence. Interestingly, lake 20198 displayed the highest average nematode burden within the basin at 28 per frog, while lake 20197 averaged less than 1 nematode per frog. It may be hypothesized that heavily diseased frogs in lake 20197 may have all died before this collection period, and thus only low nematode burdened individuals survived. 20198's large nematode presence may be a warning that heavy levels of disease are imminent.

Similar difficulty occurs when comparing sites within Sixty Lake Basin. Lakes 10 and 101 are situated approximately 1.5 kilometers apart and are significant from each other. Both are uninfected. There is no other significance with other sites at this location. Lake 10 consists of two specimens, comprising the smallest size for a lake population within this study. The power of this result is thus greatly reduced. It was expected no significance would have been observed regarding the distribution of nematodes within Sixty Lake because of the lack of detectable disease. The uncertainty regarding this result is a concern due to the small sample size of lake 10. Similar to comparing Cow Creek to Milestone and Sixty Lake, this issue may be resolved by increasing the sample sizes to properly reflect a more uniform distribution of sample sizes at the lakes of collection.

The parameter of sex had no bearing upon the absence or presence of nematodes. A highly non-significant p-value = .7824 was calculated for male burdens vs. female burdens. The ratio of

males to females was approximately 1:1.Given this result, the nematode is non-discriminatory in regards to sex.

Seasonal effects were observed to help indirectly measure the parameter of size. It was believed frogs captured earlier in the summer tended to be slightly smaller than those caught in the later months of August and September. Comparison of these means revealed another non-significant p-value = .0698. The early season displayed a mean nematode number of 14 per frog and the late season displayed 10 per frog. While this is an indirect technique of measuring the parameter of size, a more accurate and desirable method would be to observe snout to vent length of each frog. Unfortunately, these values were not available for this research. It was hypothesized that larger, more mature specimens would display a higher burden of nematodes because their anatomy can support higher densities. Until a more precise means of measuring this parameter is accomplished, it cannot be said with certainty size is not significant to nematode burden.

The most significant values in this research were the locations of nematodes within the digestive tract of R. muscosa. All 3 mean values of stomach, duodenum and lower GI burden were significant values. The duodenum exhibited the highest average of 8 nematodes per frog. The stomach showed a mean of less than 1 per frog, and the lower GI carried an intermediate value of 4 nematodes per frog. The high populations of nematodes within the duodenum may reflect the small intestine's physiological function. The duodenum is the initial point of digestion and absorption. The pancreas, liver, gall bladder, and stomach contribute acids to break down macromolecules for the body to utilize (Campbell et al. 1999). Nematodes situated within this vicinity may have a competitive advantage of obtaining nutrients for survival over those in other locations of the digestive tract. According to Campbell et al. (1999), contents within the stomach have not yet significantly broken down, and food flowing through the lower GI and colon is generally less rich in the desired nutrients than within the small intestine. Lower burdens within the stomach and large intestine may reflect these facts.

Due to the lack of data regarding the specific infection status of each frog, correlation of disease level and nematode burden was accomplished by relating seasonal differences to infection. Detection of disease increased within Milestone in the later months relative to early summer observations. No significant value was detected by comparison of the lakes in Milestone between these time periods. This suggests nematode burdens do not necessarily increase or

decrease with prolonged exposure to the Chytrid fungus. Frogs that are infected will be characterized by a relatively high, constant nematode burden. A comparison using specific quantitative data obtained by swabbing specimens to describe specific levels of infection would be more accurate in confirming the results of this test.

The parasite family Cosmocercidae is usually characterized by a direct life cycle and is often host specific. It is normally found within the digestive tracts of both reptiles, and more commonly amphibians (Vanderburgh and Anderson, 1987).

The parasite of genus *Aplectana* is within Cosmocercidae and is also understudied and possesses limited literature in scientific descriptions. It has been described from select frog species within North and South America. Organisms such as the horned leaf frog in Brazil, *Proceratophrys appendiculat* (Boquimpani-Freitas et al., 2001), *Bufo boreas* in southern California (Bursey, 1999), and Great Plains narrow-mouthed toad, *Gastrophyrne olivacea* in Arizona have displayed species within the genus Aplectana that are often situated within the intestine of amphibian hosts (Bursey and Cheam, 1998). These worms may play a role in deterring nutrient absorption within the intestine. This may affect food intake and overall diet patterns. Due to the isolated location of the study sites, it may be likely more species of *Aplectana* or other genre may be discovered after further identification of the nematode samples. General observation of the total nematode population revealed a higher female to male ratio of specimens within the sample size. This may suggest proliferation of the parasite life cycle. Large quantities of nematodes are consistently reproducing without the requirement of an intermediate host. Observation from digestive tissues of heavily nematode burdened frogs also revealed inflammation and stress of the tract linking, likely originating from the nematode's occurrence.

The purpose of this research was an attempt to confirm or reject the establishment of a nematode to *R. muscosa.* While it is difficult to determine whether disease causes nematode presence, or vice versa, based on the findings of this study, greater nematode burdens are linked to disease. Burdened frogs are also likely to display the greatest density of worms within the duodenum due to nutrient absorption. Most other parameters investigated within this research proved to be non-significant and are not concerns regarding this parasite. Personal bias was a worry at the origin of this research, but was resolved as well as possible by following blind experimental methods. The biggest problem within this study may have stemmed from non-consistent sample sizes. While some test populations exhibited over 80 samples, some only

possessed 2. This greatly affected the power and confidence with which conclusions could have been made concerning results of many statistical tests. While this matter could not be resolved within the scope of this research, future studies will be well served with more consistently large sample sizes.

The inability to completely comprehend the mortality of amphibians is a conflict that is currently being elucidated. The additional information provided by correlations presented here will help build the understanding to the health concerns *R. muscosa* currently faces. This will establish another parameter that may prove essential to conservation of this species.

Acknowledgements

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