An Investigation of the Effects of the Parasitic Nematode Aplectana hamatospicula on the Performance and Behavior of Cuban Treefrogs (*Osteopilus septentrionalis*)

Kerri Surbaugh

*University of South Florida, Kerri.Surbaugh@Gmail.com*

Follow this and additional works at: [https://scholarcommons.usf.edu/etd](https://scholarcommons.usf.edu/etd)

Part of the Evolution Commons, and the Other Education Commons

**Scholar Commons Citation**


[https://scholarcommons.usf.edu/etd/7962](https://scholarcommons.usf.edu/etd/7962)

This Thesis is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
An Investigation of the Effects of the Parasitic Nematode *Aplectana hamatospicula* on
the Performance and Behavior of Cuban Treefrogs (*Osteopilus septentrionalis*)

by

Kerri Surbaugh

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Integrative Biology
College of Arts and Sciences
University of South Florida

Major Professor: Jason Rohr, Ph.D.
Loren Cassin Sackett, Ph.D.
Andrew Kramer, Ph.D.

Date of Approval:
June 21, 2019

Keywords: Anorexia, Ecological Immunology, Sickness Behavior, Anuran, Helminth, Performance Metrics

Copyright © 2019, Kerri Surbaugh
# TABLE OF CONTENTS

Table of Contents ............................................................................................................. i

List of Tables .................................................................................................................... ii

List of Figures .................................................................................................................. iii

Abstract ........................................................................................................................... iv

Introduction ..................................................................................................................... 1

Materials and Methods .................................................................................................... 5
  Collection and Care of Nematodes and Tadpoles ....................................................... 5
  Tadpole Exposures to Nematodes ............................................................................... 6
  Study Maintenance ...................................................................................................... 6
  Jumping Assay and Necropsy ................................................................................... 8
  Software and Statistical Methods ............................................................................. 8

Results .......................................................................................................................... 11

Discussion ...................................................................................................................... 18

References ..................................................................................................................... 24

Appendix 1: Letter of IACUC Approval .......................................................................... 32
LIST OF TABLES

Table 1: Results of GLM analyses for differences in mass and total length gain over the larval period, days required post-infection for tadpoles to mature to Gosner stage 42 (metamorphosis), mass gain from Gosner stage 42 to the jumping assay, and performance on the jumping assay....... 15
LIST OF FIGURES

Figure 1: Partial residuals of the GLMs fitted with Gaussian distributions are displayed with the fitted line in black and the 95% confidence bands indicated in gray ............................................................................................ 14

Figure 2: Partial residuals of GLMs fitted with binomial distributions are displayed with the fitted line in black and 95% confidence bands indicated in gray ............................................................................................ 16

Figure 3: Results of path analysis (SEM, z = -0.099, p = 0.921) showing no indirect effect of infection status on frog jumping performance relative to control. ...................................................................................................... 17
ABSTRACT

Parasitic infections are ubiquitous in nature, and host-parasite dynamics can have powerful effects on wildlife populations. Many species have evolved behavioral responses to infection that can help mitigate damage from parasites. Anorexia is a common response to infection observed throughout the animal kingdom. Reducing nutrient intake can help shift host resources from digestion to immunity, as well as limit resources available to parasites. Reduced feeding can weaken the host, but in some host-parasite interactions, this cost is less than that of maintaining an infection. Here, I describe an experiment aimed to explore the effects of the parasitic nematode *Aplectana hamatospicula* on the Cuban treefrog (*Osteopilus septentrionalis*) across life stages. Tadpoles were exposed to *A. hamatospicula* larvae or a sham exposure and growth and behavior were quantified. After metamorphosis, the jumping performance of these frogs was assessed. I revealed that *A. hamatospicula* could infect and complete its lifecycle in tadpoles. This infection was unique in that it persisted through metamorphosis with the worm continuing to reproduce in the intestinal tract of the terrestrial frogs. These infections reduced the relative mass gain of tadpoles. However, post-metamorphic frogs were able to compensate for this lower growth when provided an *ad libitum* diet, and infection did not directly or indirectly impact jumping performance, perhaps because of this compensation. Tadpoles that prevented or cleared the infection had a higher rate of anorexia, suggesting that anorexia might be a successful disease-mitigation response to *A. hamatospicula*. 
INTRODUCTION

Wildlife are continuously confronted with parasites, and these interactions can have immense effects on wildlife populations (Tompkins and Begon 1999, Tompkins et al. 2011, Watson 2013). Although infections have been listed as one of the top five causes of wildlife extinctions in the United States, host-parasite dynamics are not yet well understood (Wilcove et al. 1998, Bower et al. 2019, Sarabeev et al. 2019). As we enter a period of biodiversity loss unparalleled in recorded history, understanding and managing parasitic diseases is proving to be imperative (Ceballos et al. 2015, Cable et al. 2017). Amphibians have proven especially vulnerable to habitat loss, climate change, pollution, invasive species introduction, and the resulting impacts these factors have on infectious disease (Jenkinson et al. 2018, Rumschlag and Rohr 2018, Cohen et al. 2019). Understanding the parasitic challenges that amphibians face, as well as their responses for coping with infections, could be critical to their successful management and conservation (Bower et al. 2019).

There are many host behavioral changes in response to parasitic infection that have been observed across vertebrate taxa. For example, animals can practice self-medication as a means to combat parasitic infections (Poulin 1995, Lozano 1998). Lambs with high intestinal parasite loads selectively consumed more tannins, while non-infected controls showed no such predilection. As the worm burdens decreased, so too did the preference for tannins (Lisonbee et al. 2009). Chimpanzees have also been
observed self-medicating by swallowing rough leaves whole and chewing bitter pith to treat a suite of gastrointestinal helminth infections (Glander 1994, de Roode et al. 2013). Yet other changes in the feeding preferences of infected animals can be more subtle. Lambs infected with intestinal nematodes selected a higher protein diet than non-infected controls (Kyriazakis et al. 1994). Lambs receiving a protein infusion into their fourth stomachs did not experience an increase in worm burdens whereas those receiving glucose did (Brown et al. 1991). Manipulation of nutrient intake can play an integral role in parasite pathogenicity. In fact, anorexia, or reducing energy intake, may be one of the most widespread responses to parasitic infection, occurring in taxa as diverse as lambs and calves, humans, and crickets (Symons and Hennessy 1981, Hart 1988, Kyriazakis et al. 1998, Adamo et al. 2010). In many cases, anorexia serves as an effective strategy for decreasing parasitic intensity.

Amphibians are now considered to be the most threatened of all vertebrate taxa and many of their declines have been associated with infectious disease, yet little is known about their behavioral responses to infections (Stuart et al. 2004, Chikhlyaev and Ruchin 2014, Ceballos et al. 2015, Bower et al. 2019). *Aplectana* is a large and diverse genus of nematodes that parasitizes the large intestine of amphibians and reptiles on every continent except Antarctica (Chen 1966, Vhora and Bolek 2013, Ortega et al. 2015, Pun and Maharjan 2016, Sou and Sow 2018, Teixeira 2018). A recent survey in Flatwoods Park, Thonotosassa, Florida showed that over 80% of Cuban treefrogs (*Osteopilus septentrionalis*) were infected with *Aplectana hamatospicula* (Roznik, unpublished data). This nematode can be found in the intestinal tract of adult amphibians, shedding its offspring as L1 juvenile nematodes into the lumen of the gut to
be released into the environment with the feces of the host (Vhora and Bolek 2013). Juvenile worms remain in the soil and water until they develop to their infective stage (L3) (Anderson 2000). Worms then penetrate the skin of new hosts and travel to the large intestine where they complete their life cycle (Knutie et al. 2017).

This thesis investigates the following research questions. i.) Can A. hamatospicula infect and complete its lifecycle in tadpoles? ii.) Assuming that A. hamatospicula infects tadpoles, what are the consequences of these infections on host survival, growth, and development? iii) Are there changes in host behaviors that serve as defenses against or compensatory responses to these infections? iv.) Do tadpoles retain these infections through metamorphosis and into their terrestrial life stage? v.) Does infection as tadpoles impact performance of post-metamorphic frogs?

Given these questions, I had the following hypotheses. First, I hypothesized that A. hamatospicula can infect tadpoles in addition to adult frogs because the juveniles can develop to the infectious stage in water (personal observation). Second, Aplectana spp. reduces growth in adult treefrogs (Knutie et al. 2017), therefore I hypothesized that infection would affect survival, growth, and development in tadpoles. Third, as sickness behavior responses are well conserved across vertebrate taxa, I hypothesized that tadpoles would display behavioral defenses to infection. Post-metamorphic common toads infected with the lung nematode Rhabdias bufonis displayed anorexia until worm burdens fell substantially (Goater and Ward 1992). And, Knutie et al. (2017) found that post-metamorphic Cuban treefrogs could compensate for infection when provided a high resource diet. Therefore, I predicted that the Cuban treefrog tadpoles would display anorexia until worm burdens fell to a manageable level and that juvenile frogs would
compensate for energy lost from infections if provided sufficient resources. My fourth hypothesis was that *A. hamatospicula* infections would persist through and past metamorphosis. The intestinal nematode *Gyrinicola batrachiensis* can only complete its lifecycle in the herbivorous bullfrog tadpole gut, and not through the transition to that of a carnivorous frog (Pryor and Bjorndal 2005). However, Cuban treefrog tadpoles are omnivorous and the physiology of the adult and tadpole gut should not be as dichotomous as bullfrog tadpoles (Babbitt and Meshaka Jr 2000, Smith 2005). Finally, I hypothesized that *A. hamatospicula* would negatively affect the performance of post-metamorphic frogs—especially if infections persisted through metamorphosis.
MATERIALS AND METHODS

Collection and care of nematodes and tadpoles

Sixteen adult Cuban treefrogs (Osteopilus septentrionalis) were collected from Flatwoods Park near Thonotosassa, Florida (28°07'23.1"N 82°19'08.5"W). Frogs were euthanized, necropsied, and examined for parasites using a dissecting light microscope. Frogs that were parasitized only with Aplectana spp. were selected for worm collection for the experiment. Aplectana spp. nematodes were further identified morphologically as Aplectana hamatospicula due to the hook-like spicule cap of the male nematode in the sample (voucher number to come). A. hamatospicula worms and the intestinal contents were collected from these frogs and suspended in DI water in standard 9 cm petri dishes. Intestinal contents from the frogs that were found to be free of any helminth or Opalina spp. infection were placed in petri dishes sealed with parafilm and kept refrigerated to be used as juvenile nematode food over the following weeks.

Each petri dish of nematodes and gut contents was sealed with parafilm and stored in a dark cabinet at 22°C for three weeks to permit the gravid female worms to shed the L1 juveniles and to allow these juveniles to mature to the infectious L3 stage. Cuban treefrog hatchlings were collected from rainwater tanks at the University of South Florida Botanical Gardens (28°03'31.7"N 82°25'25.9"W). Tadpoles were maintained in
the laboratory in 10-gallon aquaria filled with rainwater and fed spirulina and fish flake agar *ad libitum* for 5 days at 22°C and a 12-hour light-dark cycle prior to exposures.

**Tadpole exposures to nematodes**

Seventy-six tadpoles, Gosner stages 25-26 (Gosner 1960), were selected at random and placed individually in either control or treatment specimen cups (n = 38) filled with 30 ml DI water. A dose of 30 *A. hamatospicula* L3 nematodes in 200 µl of feces water was pipetted from the culture petri dishes into each treatment cup; 200 µl of sham (helminth-free feces water) was pipetted into the control cups. Tadpoles remained in specimen cups for 30 hours, after which each was transferred to individual deli cups filled with 325 ml pond-water. The water remaining in each treatment cup was examined under a dissecting light microscope and the number of remaining juveniles was documented.

**Study maintenance**

Tadpoles were maintained at 22°C with a 12-hour light-dark cycle for the remainder of the study. The diet was reduced by 33% from the quantity of spirulina-fish flake agar Cuban treefrog tadpoles elected to eat when food was offered *ad libitum*. Tadpoles were fed in the individual deli-cups in which they were housed. Any uneaten food was noted, removed, and replaced with fresh agar daily. Tadpoles that did not finish their food were noted as anorexic for that day. Tadpole mass and total length were recorded during each weekly water change. Tadpoles were netted, placed on a
dry screen to reduce the unnecessary transfer of water, then dropped into a tared petri dish filled with DI water to obtain their mass each week. The petri dish, complete with water and tadpole, was then placed on a laminated 1/4” grid background, which was marked with the unique tadpole ID, and photographed. After a tadpole was photographed, it was returned to its deli-cup with fresh pond water. Thirty-two days after tadpoles were exposed to the juvenile nematodes, 5 ml water samples were taken from four randomly selected deli-cups of the treatment cohort and examined under a light microscope for the presence of L1 nematodes.

Tadpoles received an additional weighing on the day of forearm emergence (Gosner stage 42) and the date was recorded as the metamorphosis date. Between Gosner stage 42 and 46, juvenile frogs were kept in a semi-aquatic environment; 350 ml deli-cups were filled with 100 ml of pond-water with moistened paper towels to provide a platform. After metamorphs completed tail absorption, the juvenile frogs were transitioned to fresh deli-cups. Each cup contained two crumpled, moistened paper towels, which were changed weekly. To determine if juvenile frogs could compensate for growth loss due to infection, the post-metamorphic frogs were fed a high resource diet: 7 1-week-old crickets, dusted with Rep-Cal Herptivite and Calcium with VIT.D₃ powders, were provided to the frogs twice weekly. Juvenile frogs were kept in these terrestrial deli-cups until all frogs had metamorphosed. Three days before the jumping assay, feces from 20 of the 38 frogs of the treatment cohort were collected and examined under the light microscope for the presence of L1 nematodes.
**Jumping assays and necropsies**

To assess whether infection with the nematodes had any residual effects on performance, five jumps from each juvenile frog were measured. The jumping assay was conducted for all frogs on the same day. Frogs were placed individually on a sheet of plastic that was taped to the floor in a long corridor kept at 25°C. After each frog was released, the distances of the first five jumps were marked and measured. Frogs reluctant to jump were prompted by a gentle nudge to the urostyle (Roznik et al. 2018). The weight and snout-vent length of each frog was recorded before being euthanized. Frogs were euthanized with an overdose of 20% benzocaine antiseptic gel spread across their head and dorsal surface. The digestive tract of each frog was removed and observed under a light microscope to quantify nematode prevalence and abundance. Frogs that had been exposed to the infectious nematodes in the beginning of the study were subdivided into two categories: those that were infected at the time of necropsy were categorized as “infected” and frogs that showed no evidence of infection upon necropsy were categorized as “exposed”.

**Software and statistical methods**

ImageJ was used to measure the total tadpole length each week, using the photographs taken with the calibrated background during weekly water changes. RStudio version 1.1.442 was used for all statistical analyses (R Core Team 2018). Change in tadpole and frog mass, as well as total tadpole length and frog snout-vent length, were calculated as a percent gain from the initial values and compared using generalized linear models (GLM) with Gaussian errors. The Anova function in the car
package was used to run the log-likelihood ratio tests, which provided the $\chi^2$ and p-values (Fox and Weisberg 2011). Tukey’s contrasts of the multiple comparison of means was conducted using the glht function in the multcomp package (Hothorn et al. 2008). Total mass gain over the larval period from exposure to metamorphosis was square root transformed for normality. Differences in the days that tadpoles required to metamorphose were evaluated using GLM with a Poisson error distribution. The Poisson error distribution model was selected over the negative binomial model by AIC comparison.

Larval development was binned into three stages for the purposes of evaluating anorexia: the first 14 days after worm exposure, the growth period following the first fourteen days, but before the days of fasting just before metamorphosis, and finally, these days of fasting before metamorphosis. Each tadpole was scored daily as showing anorexia (failed to consume the food) or not (consumed all of the food). Differences in anorexia between cohorts were compared using GLMs with binomial errors and Tukey contrasts.

To test for compensation for infection, the slopes of juvenile frog growth were compared using the lmer function from the lme4 package by fitting a mixed-effects model to the frog mass data before and after the ad libitum feeding period (Bates et al. 2015). The frog mass at metamorphosis and the frog mass at the time of jumping were taken as repeat measures, with frog as a random variable, and an interaction term between cohort and mass measurements. The direct effects of infection on jumping performance were also evaluated using the lmer function by fitting a mixed-effects model to the jumping data. Jumps 1 – 5 were taken as repeated measures, with frog as
a random variable and mass as a covariate. Indirect effects of infection on performance were evaluated using the sem function (structural equation modeling) of the lavaan package (Rosseel 2012). The path model had one direct and two indirect paths from infection status (infected or not) to average jumping distance. The two indirect paths went through Tadpole Growth (larval mass gain) and Jumping Mass (the size of frog at the time of the jumping assay), respectively.
RESULTS

All 76 tadpoles survived the L3 nematode or sham exposure, as well as the transition to individual deli-cup housing. Only six of the 38 treatment cups had any L3 nematodes remaining, and no cup contained more than one L3 nematode after the exposure period, resulting in a >99% penetration rate of the host. There were three mortalities in the control cohort due to human error during water changes, and these tadpoles were removed from the analyses ($n = 35$). Of the 38 tadpoles exposed to *A. hamatospicula*, 10 frogs (26.3%) remained infected at the end of the experiment, with an average intensity of 1.1 adult worms per juvenile frog. The 28 frogs that were exposed but not infected upon necropsy are referred to as “exposed”, whereas the 10 frogs that were infected upon necropsy are referred to as “infected”. Of the four randomly selected water samples taken from the treatment tadpole deli-cups, one showed the presence of fresh L1 nematodes. Of the 20 randomly selected fecal samples from juvenile frogs that were examined under the light microscope three days before the jumping trials, five showed the presence L1 nematodes, indicating infections that persisted through metamorphosis.

The total proportional mass gain over the larval period from exposure to metamorphosis was significantly different between cohorts (GLM, $\chi^2(2) = 16.284$, $p < 0.001$, Figure 1b). Infected metamorphs gained the least mass, followed by the exposed metamorphs, and the control metamorphs gained the most mass over the larval period. Tukey pairwise comparison showed significant differences between infected and control
tadpoles (Tukey’s HSD: p < 0.001) and exposed and control tadpoles (Tukey’s HSD: p = 0.042). The difference in mass gain between the infected and exposed tadpoles was marginally non-significant (p = 0.092). Mean percent total tadpole length change did not vary significantly between cohorts over any of the weeks of the larval period (Table 1). Percent mass change one week after exposure to nematodes varied among cohorts (GLM, $\chi^2(2) = 15.971$, p < 0.001, Figure 1a). Exposed tadpoles gained significantly less mass than control tadpoles during this week (Tukey’s HSD: p < 0.001). Infected tadpoles gained less mass than control tadpoles during this week as well, but the comparison was marginally non-significant (Tukey’s HSD: p = 0.067). There was no difference in mass gain between the exposed and infected cohorts in the first week (Tukey’s HSD: p = 0.870). No other individual week showed significant differences among the cohorts in proportional mass gain. There were no differences among the cohorts in time to metamorphosis (GLM, $\chi^2(2) = 0.317$, p = 0.853).

Exposed tadpoles (which had been exposed to the nematodes and averted infection) displayed greater anorexia (GLM, $\chi^2(2) = 14.260$, p < 0.001) than either control or infected tadpoles (p = 0.002 and p = 0.033, respectively). In the first two weeks after exposure, when the worms were establishing infection, control and infected tadpoles were less anorexic than exposed tadpoles (Figure 2a, p < 0.001 and p = 0.008, respectively). But this was no longer the case during the intermediate tadpole growth period that followed (GLM, $\chi^2(2) = 9.450$, p = 0.009, Figure 2b). Instead, control tadpoles displayed marginally more anorexia than the exposed cohort (p = 0.031). There was no difference in anorexia between any of the cohorts just before metamorphosis (GLM, $\chi^2(2) = 0.582$, p = 0.748), as all tadpoles fast during this stage.
On the high resource diet that followed metamorphosis, infected frogs gained proportionally more mass (GLM, $\chi^2(2) = 7.411, p = 0.025$) than either the control or the exposed frogs ($p = 0.025$ and $p = 0.031$, respectively). The slopes of juvenile frog growth between the time of metamorphosis and that of the jumping assay were significantly different (GLMM, $\chi^2(2) = 7.141, p = 0.028$) with infected frogs gaining more mass than either the control frogs or those that averted infection ($p = 0.008$, and $p = 0.042$, respectively). Control frogs and those that averted infection had slopes of growth that did not differ significantly ($p = 0.485$). Infection was not found to have a direct effect on jump distance (GLMM, $\chi^2(2) = 1.319, p = 0.517$). And although a path analysis showed that infection reduced tadpole growth (SEM, $z = -4.459, \beta = -0.554, p < 0.001$) and that there was a positive relationship between frog body size at the time of jumping and average jump distance (SEM, $z = 4.216, \beta = 0.532, p < 0.001$), there was no indirect effect between infection and average jump distance (SEM, $z = -0.099, \beta = -0.015, p = 0.921$, Figure 3).
**Figure 1.** Partial residuals of the GLMs fitted with Gaussian distributions are displayed with the fitted line in black and 95% confidence bands indicated in gray. Panel (a) shows tadpole growth, in terms of mass, over the first week post-exposure to the *A. hamatospicula* nematodes. Panel (b) shows tadpole growth, in terms of mass, over the larval period from date of exposure to that of achieving Gosner stage 42 (metamorphosis). Significant cohort comparisons are indicated by differing letters. Plots were created using the R package visreg.
Table 1. Results of GLM analyses for differences in mass and total length gain over the larval period, days required post-infection for tadpoles to mature to Gosner stage 42 (metamorphosis), and performance on the jumping assay. Comparisons significant at the 95% level are bolded and marginally non-significant p-values are noted in brackets. Pairwise comparisons not shown were not found to be significant. "c" indicates the control cohort, "e" indicates the exposed cohort, and "t" indicates the cohort which remained infected. Means and standards errors (s.e.) were calculated from raw data. Gaussian error distributions were used for all analyses with the exception of "days from exposure to Gosner stage 42", for which a Poisson distribution was used.

<table>
<thead>
<tr>
<th>metrics</th>
<th>mean ± s.e. across cohorts</th>
<th>effect of treatment type</th>
<th>Tukey post hoc tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>exposed</td>
<td>infected</td>
</tr>
<tr>
<td>tadpole metrics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>delta mass week 1 (%)</td>
<td>133.326 ± 8.037</td>
<td>95.363 ± 5.490</td>
<td>101.133 ± 9.455</td>
</tr>
<tr>
<td>delta mass week 2 (%)</td>
<td>26.770 ± 1.816</td>
<td>27.750 ± 2.251</td>
<td>22.621 ± 2.272</td>
</tr>
<tr>
<td>delta mass week 3 (%)</td>
<td>16.024 ± 1.573</td>
<td>19.217 ± 1.816</td>
<td>11.319 ± 3.036</td>
</tr>
<tr>
<td>delta mass week 4 (%)</td>
<td>9.989 ± 2.561</td>
<td>10.885 ± 2.493</td>
<td>5.973 ± 5.826</td>
</tr>
<tr>
<td>delta mass week 5 (%)</td>
<td>0.047 ± 0.048</td>
<td>0.013 ± 0.034</td>
<td>-0.010 ± 0.112</td>
</tr>
<tr>
<td>delta SVL week 1 (%)</td>
<td>29.851 ± 1.225</td>
<td>24.932 ± 1.675</td>
<td>26.839 ± 3.422</td>
</tr>
<tr>
<td>delta SVL week 2 (%)</td>
<td>0.103 ± 0.008</td>
<td>0.103 ± 0.012</td>
<td>0.104 ± 0.035</td>
</tr>
<tr>
<td>delta SVL week 3 (%)</td>
<td>0.083 ± 0.010</td>
<td>0.086 ± 0.011</td>
<td>0.069 ± 0.039</td>
</tr>
<tr>
<td>delta SVL week 4 (%)</td>
<td>0.051 ± 0.008</td>
<td>0.069 ± 0.012</td>
<td>0.059 ± 0.042</td>
</tr>
<tr>
<td>delta SVL week 5 (%)</td>
<td>0.069 ± 0.013</td>
<td>0.055 ± 0.013</td>
<td>0.102 ± 0.021</td>
</tr>
<tr>
<td>total mass gain (%)</td>
<td>157.033 ± 9.370</td>
<td>126.419 ± 10.451</td>
<td>87.796 ± 7.840</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>days from exposure to Gosner stage 42 (count)</td>
<td>33.086 ± 0.908</td>
<td>33.107 ± 0.827</td>
<td>32.00 ± 1.633</td>
</tr>
</tbody>
</table>

jumping assay

<table>
<thead>
<tr>
<th></th>
<th>mean ± s.e. across cohorts</th>
<th>effect of treatment type</th>
<th>Tukey post hoc tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>exposed</td>
<td>infected</td>
</tr>
<tr>
<td>jump 1 distance (cm)</td>
<td>16.200 ± 0.780</td>
<td>15.593 ± 1.397</td>
<td>18.850 ± 1.726</td>
</tr>
<tr>
<td>jump 2 distance (cm)</td>
<td>18.243 ± 1.018</td>
<td>16.148 ± 1.099</td>
<td>14.800 ± 1.841</td>
</tr>
<tr>
<td>jump 3 distance (cm)</td>
<td>16.943 ± 0.880</td>
<td>15.963 ± 0.999</td>
<td>16.150 ± 1.863</td>
</tr>
<tr>
<td>jump 4 distance (cm)</td>
<td>16.286 ± 0.968</td>
<td>15.685 ± 1.142</td>
<td>17.500 ± 1.938</td>
</tr>
<tr>
<td>jump 5 distance (cm)</td>
<td>16.300 ± 0.980</td>
<td>14.130 ± 1.249</td>
<td>15.700 ± 2.230</td>
</tr>
<tr>
<td>mean jump distance (cm)</td>
<td>16.794 ± 0.689</td>
<td>15.504 ± 0.970</td>
<td>16.600 ± 1.757</td>
</tr>
</tbody>
</table>
Figure 2. Partial residuals of the GLMs fitted with binomial distributions are displayed with the fitted line in black and 95% confidence bands indicated in gray. Panel (a) shows the log odds of anorexic days observed in the first 14 days after exposure to the A. hamatospicula nematodes (GLM, $\chi^2(2) = 42.039, p < 0.001$). The tadpoles that averted infection displayed more anorexia than either the control tadpoles or the tadpoles that remained infected. Panel (b) shows the log odds of anorexia after the first two weeks (GLM, $\chi^2(2) = 9.450, p = 0.009$). Control tadpoles were marginally more anorexic during the intermediate growth period than the tadpoles that were exposed, but averted infection. Significant cohort comparisons are indicated by differing letters. Plots were created using the R package visreg.
Figure 3. Results of path analysis (SEM, $z = -0.099$, $p = 0.921$) showing no indirect effect of infection status on frog jumping performance relative to control. Probabilities and standardized coefficients are shown directly next to each path.
DISCUSSION

As hypothesized, *A. hamatospicula* was able to infect and complete its lifecycle in the tadpoles, with some tadpoles retaining the infection through metamorphosis. Exposure to the parasite negatively impacted growth regardless of whether the tadpole retained the infection or not. Tadpoles that were exposed, but averted infection, gained significantly less mass than control tadpoles, and infected tadpoles gained the least mass. But when provided a diet of crickets *ad libitum*, the infected juvenile frogs compensated for the infection by gaining more mass than either the control tadpoles or those that were exposed but averted infection. This accelerated growth equalized the cohorts so that there was no difference in mass at the time of the jumping assay, reducing any indirect effect on performance that may have been mediated through frog size. Tadpoles that were exposed to the nematodes, but averted infection, displayed anorexia in the first two weeks after exposure.

Anorexia is a common symptom of parasitic infection (Symons and Hennessy 1981, Kyriazakis et al. 1998, Adamo et al. 2010, Poon et al. 2015). However, previous observations of disease-induced reduction of feeding in tadpoles have been attributed to a physical impediment to feeding rather than an evolved or learned behavioral change. The parasitic chytrid fungus, *Batrachochytrium dendrobatidis* consumes the keratinized mouthparts of tadpoles, causing deformation, and a subsequent reduction of feeding rates (Blaustein et al. 2005, DeMarchi et al. 2015). Ranavirus dissolves the
internal organs of amphibians, eliciting anorexia in infected tadpoles (Smith et al. 2007, Cunningham et al. 2008, Gray et al. 2009, Wang et al. 2011, Meng et al. 2014, Miller et al. 2015). But it is unlikely that the relatively low dose of microscopic L3 nematodes in this study could have obstructed feeding, as the subset of tadpoles that retained the infection never displayed anorexia.

Anorexic behavior can be more than a symptom of damaged organs or general malaise. A tradeoff between immunity and digestion has been noted across the animal kingdom (Hart 1988, Tizard 2008). Knutie et al. (2017) found that post-metamorphic frogs that were fed a low resource diet during the establishment phase mounted a higher humoral IgY response—a response that was suggested to have contributed to relatively fewer Aplectana spp. worms establishing compared to the frogs on a high resource diet (Knutie et al. 2017). The anorexia observed in this study may have also contributed to a higher immunological defense. The tadpoles that averted infection displayed anorexia during the first two weeks after exposure, during the establishment phase of Aplectana (Knutie et al. 2017). These same tadpoles ceased to display anorexia after the worm establishment period. Instead, they were found to be less anorexic than the control tadpoles in the intermediate growth period. After the worms were prevented from establishing, the tadpoles resumed normal or compensatory rates of feeding.

As hypothesized, tadpoles that remained infected throughout the larval period gained less mass than the control tadpoles. These infected tadpoles gained less than the anorexic, worm-free tadpoles as well, although this difference was marginally non-significant. The first week post-exposure to the nematodes was a critical growth period.
for all tadpoles. And, it is unsurprising that anorexia at this time would create a significant difference in growth between the control and anorexic tadpoles. However, the difference in mass gain between the control tadpoles and those that averted infection decreased over the duration of the larval period, apparently after the previously anorexic tadpoles resumed normal feeding practices. The comparison of gains between control and infected tadpoles had not been significantly different in that critical first week after exposure. But the difference in mass gains between the infected and control tadpoles increased throughout the larval period, in spite of similar feeding practices (Figure 1).

It appears that the short period of anorexia at the critical establishment period post-exposure was less costly to the exposed tadpoles than maintaining the nematode infection. However, exposure to the worms was injurious both to the tadpoles that tolerated the infection and those that displayed anorexia and mitigated the infection, as both cohorts gained significantly less than the control tadpoles. Contrary to our hypothesis, exposure did not prove to be stressful enough to promote a premature metamorphosis. There was no difference in the days control, infected, or exposed tadpoles required to mature.

As hypothesized, the tadpole intestinal tract proved hospitable enough of an environment for *A. hamatospicula* to complete its lifecycle, as juvenile L1 nematodes were found to have been shed into the tadpole water 32 days after infection. Unlike intestinal trematodes that encyst in ancillary tadpole tissues and migrate to the intestine after metamorphosis (Bolek and Janovy 2008, Chikhlyaev and Ruchin 2014), *A. hamatospicula* established in the tadpole gut and likely remained in the intestinal tracts through the restructuring process of metamorphosis. Unlike the nematode *Gyrinicola*
*batrachiensis*, which only parasitizes the herbivorous tadpoles and not the carnivorous adult life stage of bullfrogs (Pryor and Bjorndal 2005), the adult *A. hamatospicula* worms continued to reproduce in the newly metamorphosed gut, shedding juvenile nematodes into the lumen to be expelled by the frog's feces. Cuban treefrog tadpoles are partially carnivorous, and the physiological changes in the metamorphosed frog gut may not be as pronounced. However, given the generalist nature of *Aplectana spp.* worldwide, it could be that *A. hamatospicula* has adapted to both the aquatic as well as the terrestrial stage of amphibians, regardless of host species.

As predicted, when post-metamorphic frogs were shifted onto a high resource diet and fed crickets *ad libitum*, infected frogs utilized the additional food to compensate for the mass lost during the larval period. Infected frogs gained significantly more mass than either the control or exposed frogs. This compensation did not enable the infected frogs to become larger than control frogs at this time, but rather narrowed the difference in mass between cohorts. Contrary to my hypothesis, the presence of infection alone was not found to have a direct effect on jumping performance—even with the width of just one adult nematode being large enough to fit snugly in the juvenile frog's large intestine. The path analysis showed that infection had a negative effect on tadpole growth and that smaller frogs did not jump as far as larger frogs. But given the compensatory feeding response of the infected frogs on the *ad libitum* diet, the differences in frog masses at the time of the jumping assay were not owing to the presence of infection.

Therefore, the severity of the cost of the infections was found to depend on resource availability. Frogs could tolerate *A. hamatospicula* in an environment that
provides food *ad libitum*. However, an environment with restricted resources would make infected juvenile frogs comparatively smaller and less likely to achieve a competitive adult body size, or reproductive success (Berven and Gill 1983, Altwegg and Reyer 2003, Earl and Whiteman 2015). The timeline for Cuban treefrog development, and therefore compensation, is brief: males can achieve sexual maturity within 90 days of transformation (at only double their mass at metamorphosis), and are often dead within a year (Meshaka 2001).

Native treefrogs in Florida, Puerto Rico, and Hawaii could afford an infection even less, as they are already typically smaller than the invasive Cuban treefrog and any further reductions in size at metamorphosis would only broaden this disparity (Meshaka 2001, Rödder and Weinsheimer 2009, Mitchell and Pague 2014). Cuban treefrog females produce larger and more frequent clutches in a wet season (each clutch ranging between 1,000 and 16,000 eggs rather than 500 to 2,000), and crowd breeding pools with cannibalistic offspring that predate upon the native amphibians both before and after metamorphosis (Meshaka 2001, Wyatt and Forys 2004, Smith 2005). The superior relative size of Cuban treefrogs permit an increased range of prey selection that includes conspecific metamorphs and smaller native species (Meshaka 2001), and reduced growth of native frogs at early life stages due to *A. hamatospicula* infection would increase their odds of becoming prey rather than finding it.

Future work could illuminate whether *A. hamatospicula* is infecting the invasive Cuban treefrog or native tadpoles in wild populations. Most of the Cuban treefrogs collected from Flatwoods Park, Florida that were examined for infection showed the presence of *A. hamatospicula* worms in the hind gut. Due to protection of native
species, destructive sampling was not an option, but 16.7% of adult squirrel (Hyla squirrellia) and 30% of pinewoods (Hyla femoralis) treefrog feces examined in Flatwoods Park, Florida showed the presence of L1 nematodes (Roznik, unpublished data). But it is not clear if these infections were obtained before or after critical growth stages. This remarkably versatile parasite may have a greater impact on amphibian populations dynamics than previously believed.
REFERENCES


mismatches on chytrid fungus Batrachochytrium dendrobatidis prevalence are moderated by life stage, body size, elevation and latitude. Ecology letters.


Pryor, G. S., and K. A. Bjorndal. 2005. Effects of the nematode Gyrinicolaphydatrachiensis on development, gut morphology, and fermentation in bullfrog tadpoles (Rana


APPENDIX 1

Letter of IACUC Approval

MEMORANDUM

TO: Jason Rohr, Ph.D.

FROM: Farah Moulvi, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 5/22/2017

PROJECT TITLE: Human modified landscapes: Effects on wildlife disease risk and host population dynamics

FUNDING SOURCE: USF department, institute, center, etc.

IACUC PROTOCOL #: W IS00003798

PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 5/22/2017:

- *Bufo terrestris* (Unknown because we are proposing a capture-mark-recapture field study. Most are expected to be postmetamorphic (i.e. juveniles and adults). We expect to capture <10,000.)
- *Osteopilus septentrionalis* (Unknown because we are proposing a capture-mark-recapture field study. Most are expected to be postmetamorphic (i.e. juveniles and adults). We expect to capture <10,000.)
- *Hyla cinerea* (Unknown because we are proposing a capture-mark-recapture field study. Most are expected to be postmetamorphic (i.e. juveniles and adults). We expect to capture <10,000.)
- *Hyla gratiosa* (Unknown because we are proposing a capture-mark-recapture field study. Most are expected to be postmetamorphic (i.e. juveniles and adults). We expect to capture <10,000.)
- *Hyla femoralis* (Unknown because we are proposing a capture-mark-recapture field study. Most are expected to be postmetamorphic (i.e. juveniles and adults). We expect to capture <10,000.)
juveniles and adults). We expect to capture <10,000.)

Bufo marinus (Unknown because we are proposing a capture-mark-recapture field study. Most are expected to be postmetamorphic (i.e. juveniles and adults). We expect to capture <10,000.)

Hyla squirella (Unknown because we are proposing a capture-mark-recapture field study. Most are expected to be postmetamorphic (i.e. juveniles and adults). We expect to capture <10,000.)

Please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years, all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.**

- **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

- **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons convenience. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.

- **The PI must assist the IACUC with tracking wild animal field research activities.** The PI must report episodes of wild animal use, the approximate range of taxa, and the approximate numbers of animals encountered or used at intervals appropriate to the study but at least once a year.