ABSTRACT:
Parasites manipulate their host's behavior to benefit themselves, which has detrimental effects on the host's ability to survive or reproduce (Moore 2002). For example, parasites inhibit behaviors that could increase host mortality (Soghigian *et al.* 2017), thereby minimizing the risk of the host dying before the parasite enters its next stage of development. Alternatively, parasites may initiate behaviors otherwise absent from the host's usual repertoire, such as behaviors that facilitate the transport of the parasite to the next environment required to complete its lifecycle (Hughes 2015). Despite its relevance to the effective biocontrol of major agricultural pests and the application of how pathogens cause significant behavioral changes in animals and humans, the mechanisms behind parasite modification of host behavior are not well-understood. Here, I propose using the novel host-parasite model of parasitic horsehair worms and their field cricket host to examine whether the direct manipulation of the host's neural signaling by the parasite is responsible for the modifications to host behavior during infection, specifically those alterations that minimize host predation risk and guarantee the parasite's transfer into an aquatic environment. I plan to invest two important neural signaling molecules, octopamine and serotonin, as the causative effectors of this modulation of behavior. Together with my undergraduate researchers, we will: 1) quantify changes in host locomotor activity, courtship calling, aggression, and water-seeking behavior in infected crickets relative to healthy crickets and 2) in collaboration with [redacted] we will use High Performance Liquid Chromatography (HPLC) to quantify octopamine and serotonin levels in the brains of healthy and infected crickets at sexual maturity and parasite emergence. The proposed research will serve as important preliminary research that will increase my competitiveness to apply for external funding to support a new direction of research in my career. More importantly, this research will have wide-spread impacts on the field of behavioral ecology by increasing our understanding of the proximate mechanisms for host behavioral changes after parasitic infection, a surprisingly understudied area despite the significance of parasitism on 1) the biocontrol of pests, 2) the ecological impacts of parasite biodiversity, 3) the transmission of diseases that use parasites as vectors, and 4) the behavioral modifications after infection that lead to psychosis in humans.

I. STATEMENT OF THE PROBLEM

Every living organism harbors some form of parasite, whether it be large macroparasites that can be seen with the naked eye or microscopic organisms living on or within the body. While some parasites go unnoticed or have minimal impacts on their hosts, others have significant and detrimental effects. In the latter case, many of these impacts are due to the direct manipulations of host physiology and behavior to benefit the growth, development, and transmission of the parasite. There are many "flashy" examples of these host-parasite interactions that have been highlighted in the popular press in recent years, including emerald wasps that turn their cockroach hosts into zombies under their own control, large aquatic horsehair worms that initiate suicide behavior in crickets, and fungi that force ants to rain down infective spores onto their sisters who will soon also become infected. Although host-parasite interactions have received significant media attention (mostly to repulse their unsuspecting audience), we still have little understanding of how host behavior is actually manipulated by parasites. The purpose of this project is to expand on our understanding of the complex interactions hosts and parasites have with each other by studying the cricket–horsehair worm system. We plan to quantify behavioral differences between infected and healthy crickets and analyze host brain samples to identify the proximate mechanisms of how parasites alter their host's behaviors.

II. SIGNIFICANCE OF THE PROBLEM

Parasite modification of host behavior is something that truly has global importance multiple levels. As carbon emissions soar, climates are becoming more extreme, resulting in drastic food shortages due to the negative agricultural impacts of unexpected heat waves, expansive floods, and early/late frosts. A compounding factor is that many invasive agricultural pests actually thrive in extreme environments that wipe out their otherwise natural enemies. Scientists have developed innovative methods to combat pest species through biocontrol, which relies in the controlled release of other organisms that work to minimize the pest population. These biocontrol agents are often predators of the pests, however more recently efforts have been focused on the use of parasites as they may have fewer negative ecological ramifications for the environment. For example, parasitic nematodes are being used to control the invasive and destructive Japanese beetle that has been sweeping across the U.S. If we are to use parasites in this capacity, it is imperative that we truly understand the effects they have on their host and also have a complete understanding of their impacts on the environment to avoid unintended consequences that may be more detrimental than the pest species themselves.

Further, host manipulation by parasites is not unique to animals. It has significant implications on human health because the parasites responsible for well-known disease such as toxoplasmosis, rabies, and sleeping sickness also alter the behaviors of infected humans in detrimental and deadly ways. For example, there is a growing body of evidence suggesting a correlation between schizophrenia and toxoplasmosis (Fuglewicz *et al.* 2017), a condition resulting from exposure to an intracellular parasite commonly found in cat litter). Therefore, understanding how parasites hijack host physiology and alter host behavior has important clinical ramifications on the global scale. Identifying how behavioral modifications are induced in other organisms may lead to creative treatments that slow parasite growth or transmission in human populations, or more dramatically, prevent parasitic infection in the first place.

Finally, we are currently in the midst of our 6th mass extinction here on Earth. This is the first due to anthropogenic changes to the environment and it is having a disproportionally negative effect on the extinction of parasites. Due both in part to the fact that parasites represent the largest amount of undescribed biodiversity we have on Earth, and that parasites have specially co-evolved to live on particular hosts (oftentimes they are constrained to live on only a single species), it is currently estimated that upwards of 30% of parasites will be extinct by 2070 (Carlson *et al.* 2017). Given this impending threat, I believe we have a moral prerogative to learn and understand as much we can about these understated and ecologically important organisms before they are perhaps lost forever.

III. SUMMARY OF THE PERTINENT LITERATURE

The horsehair worm (*Paragordius various*) is a long-lived parasite that infects terrestrial invertebrates (e.g. crickets, mantids, cockroaches) during its juvenile stage. Interestingly, once mature, *P. varius* must emerge from its terrestrial host directly into an aquatic environment, which presents an interesting dilemma for the parasite. The horsehair worm is famous for inducing 'suicidal behavior' in its terrestrial hosts, where it manipulates its host to seek out and jump into open water (Thomas *et al.* 2002), a behavior that often results in host drowning and is otherwise absent in most terrestrial insects. Surprisingly, horsehair worms grow and reside in the hemocoel (i.e. the body cavity) of their host, usually within the abdomen, yet as with other animals insect behavior is coordinated in the brain – so how is the horsehair worm able to induce these behavioral alterations? Previous studies have investigated altered in brain growth (Thomas *et al.* 2003) or neural protein levels (Biron *et al.* 2005, 2006) but have been unable to come to a definitive causal mechanism of host behavioral manipulation.

Here, I propose investigating two important neural signaling molecules, octopamine and serotonin, as the causative effectors of the modulation of behavior in the cricket-horsehair worm system. Octopamine plays a strong role in mediating the 'fight or flight' response and courtship behavior in insects (Adamo *et al.* 1995), with aggressive and courting individuals exhibiting higher levels of octopamine. Alternatively, serotonin modulates the daily circadian rhythm (Saifullah & Tomioka 2002), where increases of it during daylight suppress locomotor and courtship activity at a time when predators are more abundant. These bioamines are known to directly alter host behavior in other host:parasite interactions (Adamo 2005; Libersat *et al.* 2009), and given that insects infected with horsehair worms have their behavior altered more drastically at night than during the day (Sanchez *et al. 2007*), there appears to be a circadian rhythm to the parasite-induced manipulation of behavior. If horsehair worms indeed manipulate either octopamine or serotonin in the brains of their hosts, they would be able to alter an entire suite of host behaviors to assist in their own growth, development, and emergence.

My lab has investigated the physiological, developmental, and behavioral modifications of the sand field cricket (*Gryllus firmus*) by the horsehair worm for the past two years. We have determined that infected crickets have fewer energy stores (i.e. fat), produce fewer spermatophores (i.e. sperm-containing packets that are transferred to females), and have significantly lower mating success compared to healthy crickets. This proposed project for this upcoming summer will expand on our understanding of the cricket–horsehair worm system by quantifying behaviors of infected and healthy crickets to identify differences in locomotion, reproduction, aggression, and water-seeking activities; and then analyzing host brain samples to identify the proximate mechanisms of how parasites alter their host's behaviors.

IV. RESEARCH OBJECTIVES/HYPOTHESES

Objective 1 – Behavioral Assays: Assay fundamental behaviors in healthy and infected crickets to determine how parasitic infection modulates behavior at two critical time points in the host/parasite interaction: 1) upon host maturation and 2) just prior to parasite emergence, and specifically whether these alterations benefit the survival and emergence of the parasite at a cost to the normal behavioral repertoire of the host.

a. **Approach:** We will infect host juvenile crickets approximately 2 weeks prior to molting into adults. Upon maturation (approx. 21 days post-infection) and just prior to parasite emergence (approx. 28 days post-infection), we will conduct behavioral assays to quantify levels of locomotion, courtship calling, aggression, and water-seeking in healthy and infected crickets.
b. **Predictions:**
   - *Prediction 1:* If parasitic infection alters host behavior to minimize predation risk and reduce the amount of nutritional resources being metabolized by the host, then I predict that upon host maturation we will observe lower levels of locomotion, courtship calling, and aggression in infected crickets relative to healthy crickets.
   - *Prediction 2:* If parasitic infection alters host behavior to increase the likelihood that the parasite will be able to emerge from its host into an aquatic environment, then I predict that when parasite development is complete we will observe higher levels of locomotion and water-seeking behavior in infected crickets relative to healthy crickets.

Objective 2 – Brain Assays: Quantify octopamine and serotonin levels in the brains of healthy and infected crickets upon host maturation and just prior to parasite emergence to: 1) correlate whether parasitic infection alters the levels of important neural signaling molecules in the host brain and 2) identify whether alterations in brain chemistry align with the two critical time points in the host/parasite interaction when host behaviors are most modified.

a. **Approach:** We will infect host juvenile crickets approximately 2 weeks prior to molting into adults. Upon maturation and just prior to parasite emergence, we will dissect host brain tissue to quantify the levels of octopamine and serotonin in the samples of healthy and infected crickets using High Performance Liquid Chromatography (HPLC).
b. **Predictions**
   - *Prediction 1:* If decreased octopamine levels mediate the reduction in aggression and courtship behaviors (Adamo *et al.* 1995) of infected crickets, then I predict that infected crickets will have lower levels of octopamine in their brains upon reaching sexual maturity relative to healthy crickets.
   - *Prediction 2:* If increased serotonin levels suppress locomotor and courtship behaviors (Saifullah & Tomioka 2002) in infected crickets, then I predict that infected crickets will have higher levels of serotonin in their brains upon reaching sexual maturity relative to healthy crickets, but these levels will fall below those exhibited by healthy crickets once the parasite is ready to emerge from its host (i.e. when water-seeking behavior is activated).

V. Design and Methods

A. Design of Study

This study will use juvenile male sand field crickets (*Gryllus firmus*) from the captive laboratory colony maintained by my laboratory here at Creighton University. All crickets will reared in an environmentally-controlled chamber on a 12:12 light:dark cycle maintained at 27 ºC and 80% humidity. Crickets will be randomly assigned to the 'healthy' or the 'infected' treatment, and simultaneously assigned to complete either the behavioral assays or brain assay at one of the two time points. When hosts approach either sexual maturity (~21 days post-infection) or parasite emergence (~28-days post-infection), they will be sampled according to their assigned group. Our goal is to have a minimum sample size of 20 crickets for each treatment/assay/time combination.

		Sample Time Point	
Host Treatment	**Assay**	**Host maturity (21 d post infection)**	**Parasite emergence (28 d post infection)**
Healthy	Behavioral assay	N = 20	N = 20
	Brain assay	N = 20	N = 20
Infected	Behavioral assay	N = 20	N = 20
	Brain assay	N = 20	N = 20

B. Procedures

Infection of Experimental Crickets

Crickets will be randomly assigned to either the 'healthy' or the 'infected' treatment two weeks prior to reaching adulthood. Infected crickets will be fed several milligrams of snail tissue harboring infective horsehair worm cysts. Wild *Physa sp.* snails will be collected from a small stream near Waverly, NE that has a high incidence of encysted snails (Barquin *et al.* 2015). Alternatively, healthy crickets will be fed *Physa sp.* tissue obtained from non-encysted snails collected from local aquaria stores.

Behavioral Assay Overview

The courtship calling assay will be done first, and the order of the three remaining behavioral assays will be randomly conducted afterwards. We will use assays of locomotor activity (Balenger & Zuk 2015), courtship calling (Kerr *et al*. 2010), aggression (Judge & Bonanno 2008), and water-seeking behaviors (Thomas *et al.* 2002) that have been proven effective in previous work assaying cricket behavior. A review of each assay is listed below; detailed methods for each can be found in the articles referenced above. Each assay will be conducted under red-light conditions and in an isolated room after crickets are provided 5-min of acclimation to the test arena.

- *Locomotor Behavioral Assay*: Test crickets will be placed in a 1.5 m^2 arena with a grid of boxes 0.25 m^2 boxes drawn across the floor. The number of boxes that each cricket crosses into in the 10-min assay will be recorded as the locomotive index for that individual. We will also record the latency for male movement for each individual.
- *Courtship Calling Behavioral Assay*: Test crickets will be placed in a small arena partitioned into two isolated sides. On one side we will place our male test cricket, and on the other side we will place a sexually receptive female. Males will be able to see and smell the female, but the partition will ensure no physical contact. We will then record the proportion of time that a male calls within a 4-h period as measured by the number of 5-min intervals in which the male called.
- *Aggression Behavioral Assay*: Test crickets will be placed in a divided arena with a size-match male opponent. After the 5-min acclimation period, the divider will be removed and the resulting interaction will be videotaped for 10-min. Recordings will then be scored for the latency until first contact, maximum level of aggression reached using an established cricket aggression scale (Hoffman & Schildberger 2001), and the contest winner.
- *Water Seeking Behavioral Assay*: Test crickets will be placed in a Y-maze where one end terminates in a small terrestrial arena and the other end terminates in a trough filled with water. Crickets will be placed in the center for 5 min, then dividers will be removed so the cricket can move freely within the maze. Maze arm choice (terrestrial or aquatic) will be recorded, and crickets in the aquatic arm will be recorded if they jump into the water.

Dissection and Analysis of Cricket Brains

Cricket brains will be dissected in my lab at Creighton University and stored at -80 ºC until later analysis. Once all tissues have been collected, my student Amanda and I, along with our collaborator [redacted], will quantify the levels of octopamine and serotonin in each sample using HPLC. [redacted] is an expert at analyzing biogenic amine levels in the preserved neural tissue of insects (Bubak *et al*. 2019) and mammals (Hassell *et al.* 2019) and will provide the expertise to accurately quantify the levels of octopamine and serotonin in our samples.

C. DATA ANALYSIS

All data analysis will be conducted using the statistical computing program R. I anticipate analyzing octopamine and serotonin levels in the brains of healthy and infected crickets using ANCOVAs, where intensity of infection (i.e. how many horsehair worms had infected the host) is included as a covariate. Behavioral assays for locomotion, courtship calling, aggression, and water-seeking behaviors between healthy and infected crickets will likely require non-parametric analyses as behavioral data frequently violates the basic assumptions of parametric tests. The specific analyses needed therefore cannot be predicted until I know more about the quality and distribution of data collected.

D. INVOLVEMENT OF UNDERGRADUATE STUDENTS

This collaborative project will involve 3+ undergraduates ([redacted]). I am dedicated to mentoring undergraduate research students, as evidenced by the fact that since I started accepting students into my lab in 2017, I have mentored 13 students (all for a minimum of one year, some for as many as 3.5 years) and I have helped five write proposals for summer research funding (all were successful). I help all my students experience research from the conception of a project all the way through to publication. I encourage them to use creativity to design projects that suit their own interests, then help them seek funding, learn laboratory techniques, analyze their own data, and disseminate their results through presentations and publication. This project is no exception, as it was inspired by the work of [redacted] and designed by students [redacted] to suit their interests in neuroscience. Collectively, my students have given nine off-campus, 26 on-campus presentations, and have won six competitive awards for their posters and talks. In regard to publications, I am currently mentoring three former/current students ([redacted]) in writing up their research as first authors, and I will begin mentoring [redacted] early this spring on writing the two separate, high quality publications mentioned in the timeline below.

E. TIMELINE FOR COMPLETING THE PR ECT

1) Schedule for completing the oposed project

Time period (2020)	ivity
January – May	– rain students dissection techniques and behavioral assays. – anuscript preparation for [redacted] research on parasitic anipulation of host growth, development, and immunity.
May – August *(Fellowship period)*	– ollect sample brains for preliminary HPLC analysis and visit [redacted] to meet with collaborator [redacted] about techniques. – un behavioral assays to determine specific time points and agnitudes of behavioral modifications caused by infection. – ollect brains from healthy and infected crickets for baseline nalyses of octopamine and serotonin levels. – ubmit [redacted] manuscript for publication. – anuscript preparation for [redacted] research on parasitic ffects on host life-time reproductive fitness. – tudents and I attend and present at the 2020 Animal Behavior Society meeting in Knoxville, TN (Jul 30 – Aug 3)
September – December	– HPLC analysis of neural samples collected during summer. – Prepare for follow-up experiments directly manipulating octopamine and serotonin levels *in vivo*. – Submit [redacted] second manuscript for publication.

2.) Anticipated plans for dissemination: This work is a continuation, but significant expansion, of the current work being conducted in my lab. If awarded this fellowship, it would not only serve to expand my research program but would also provide time to publish the results of two separate manuscripts from the experiments that inspired this project. My student [redacted] will be the first author on both manuscripts. The research proposed here is part of a broader project I anticipate will be completed in the fall of 2021, after which it will be submitted for publication with student [redacted] as the first author. I aim to mentor my students to write-up their own research for publication, and while this takes considerably more time than writing the manuscripts myself, I feel it is imperative for their professional development to gain these skills. If awarded this fellowship, I would not only be able pursue a novel project that my students and I will publish upon its completion, but I will also have the time to provide quality mentorship to my student [redacted] for the publication of her two projects related to this work.

Further, I regularly attend and present my research at local, regional, and national conferences, and I encourage my students to attend and co-present with me at these meetings. Next summer, all active research students in my lab will attend the national 2020 Animal Behavior Society meeting with me in Knoxville, TN, a conference in which I have been invited to participate in a special topics symposium entitled “Down with the sickness: the behavior of infected animals”, which focuses on focus on the behavior of sick animals and the mechanisms by which these behavioral changes are adaptive to parasites or hosts. Additionally, my research students present their work annually at St. Albert’s Day, the Biology Department Research Colloquium, and the Phi Sigma Biological Honor’s Society Elevator Talk Competition.

3.) Future avenues for support: This opportunity will facilitate the acquisition of new research techniques and the collection of vital preliminary data, as well as provide time to publish the results of the undergraduate research that was vital to the conception of this project. Additionally, I have applied for a Haddix pre-tenure sabbatical for the spring of 2021 to designate time for the follow-up manipulation experiments mentioned in my above timeline. Being awarded this fellowship would provide support to fully train my undergraduates and fine-tune our techniques to maximize our productivity throughout the upcoming year. Collectively, these opportunities will increase my competitiveness when I apply for a Career grant from the National Science Foundation proposing an expanded investigation of parasite manipulation of host behavior.

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