

***Interactions between Batrachochytrium dendrobatidis
Exposure and Behavior in Plethodon cinereus***

By
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April, 2023



Allegany College

Department of Biology

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***A Senior Comprehensive Project in Partial Fulfillment of the Requirements
for a Bachelor of Science Degree from Allegheny College***

*I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code
and to maintain the integrity of both myself and the College community as a whole.*

Pledge

Approval by the Senior Thesis Committee:

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ABSTRACT

Behavior can be defined as a response to a stimulus. Pathogens are one form of stimulus that can influence the behaviors of the host. There is a discrepancy with host-pathogen interactions with some behaviors such as sociability increasing even in highly aggressive groups and exploration decreasing when infected with a pathogen. I am specifically interested in how *Batrachochytrium dendrobatidis* (*Bd*) influences the behavior of amphibians. *Plethodon cinereus* is one species of salamander that is known to clear *Bd* infections, shown to experience slight behavioral changes with limb lifting and a slight drop in body mass. The aggressive behaviors of *P. cinereus* between a non-exposed group and an exposed group over a three week span were examined using the Mirror Test. *P. cinereus* lacks the ability to recognize themselves in a mirror, as such, a mirror can be used to elicit aggressive, sociable, and exploratory behavior between salamanders without introducing another animal into the container. Behavior was shown to be significantly influenced by exposure. Exposed groups continuously showed high levels of nose taps, a method for the species to pick up chemical cues of their surroundings. Additionally, the exposed and non-exposed groups showed a general increase in flat trunk behavior, a non-exploratory and submissive behavior. The exposed group also showed a decrease in full trunk behavior, an exploratory and aggressive behavior. In conclusion, adequate evidence was collected to support that exposure to *Bd* had a large effect on decreases in higher energy, exploratory behaviors and increases in lower energy, non-exploratory behaviors within red-backed salamanders. The association between these behaviors and survival in the wild, and the direct mechanism and the actual measurement of energetic expenditure from a more physiological perspective are both future topics to consider when considering the influences of host-pathogen interactions between *P. cinereus* and *Bd*.

INTRODUCTION

Disease Ecology and Animal Behavior:

Behavior is a subject that entire classes and degrees focus on defining, observing and interpreting. The term behavior can be defined as a response to a stimulus or the result of a psychological process (Flint, 2002). However, this interpretation is quite restrictive and limiting. As such, different models and divisions are commonly used to interpret behavior. For instance, animal behavior can be split into five main traits: activity, exploration, boldness, sociability, and aggression (Réale et al. 2007). This is one manner in how behavior is classified and divided, from here behavior can be measured by defining what we mean by these traits. We cannot ask a spider to fill out a survey to measure how social they are feeling; instead we must match behaviors to one of these traits. Once behavior is measured, we can compare and examine factors that influence behavior such as infection.

Behavior can be more specifically measured through the use of behavioral and neurobehavioral tests such as the Mirror Test. Mirror tests look at the animal's ability for self-recognition as well as for measuring their resulting behaviors in response to their reflection (Brecht et al. 2020; Dalton et al. 2020). Humans and some other animals have the ability to recognize our own reflections. However, salamanders will fail to recognize their image and will instead mistake themselves for a potential competitor (Dalton et al. 2020). The mirror test has been previously used on *Gasterosteus aculeatus*, a type of fish commonly referred to as the stickleback, to measure behavioral changes in sociability and aggression when the fish were infected with a parasite. The number of attacks on the mirror occurred were measured to quantify levels of aggression. The hesitance to approach the mirror and the distance from the mirror without aggressive behavior occurring was used to measure sociability (Petkova et al. 2018).

This example using sticklebacks does primarily label sociability as a measure of lack of aggression. This could be potentially limiting in the regard that sociability can also take the form of beneficial behaviors. For instance, small birds can join together as a group to perform mobbing behaviors to drive off larger predators (Honda & Azuma 2021). This is a fascinating overlap between aggressive behavior performed by a social group within a species. Mirror tests have also been used on *Plethodon serratus*, or southern red-backed salamanders, a cousin to *P. cinereus*, to measure sociability and aggression. Specifically, they have been used when examining the Dear Enemy Phenomenon, a phenomenon in which an animal will potentially respond more favorably or with less aggression to recognizable competitors or neighbors once territory has been divided between them (Dalton et al. 2020). Measurements of behavior are important to consider when formatting a model to explain potential changes in behavior caused by different environmental or physical conditions.

Additionally, hesitancy to approach the mirror appears to be a behavior somewhat adjacent to an approach-avoidance conflict. The approach-avoidance conflict is when there are both potential positive and negative consequences to an action leading to conflicting feelings. Approach-avoidance is typically used in animal models to measure levels of anxiety (Aupperle, et al. 2015). For instance, if the approach-avoidance model were to be applied to the stickleback case, then they could be approaching the mirror for potentially “aggressive” reasons. They could be about to attack the mirror or show dominant behaviors to drive off a competitor, or they could be avoiding the mirror so as to avoid any potential harm from being the one attacked by the competitor. The hesitancy to initially approach the mirror could be interpreted as a “conflict”.

The relationship between behavioral models and infection can better pinpoint the changes in behavior potentially caused by infection. This can help with the distinction between when

behavior is repeatable and when behavior is influencing the susceptibility to infection of an individual and a population. For instance, male Carpetan rock lizards that tend toward high risk behaviors, measured in the amount of delay they experienced in leaving or running from a shelter, were also those with the highest intensities of blood parasite infections (Horváth et al. 2016). Their prior behavior could lead them to be more or less susceptible to infection because of this change in behavior. For instance, if infection is associated with increases in exploration by a host, then this could potentially increase the chance of a predator catching sight and eating the host. The predator could be the next stage for the pathogen in its life cycle, and in this manner this associated increase in exploration benefits the pathogen.

Additionally, if infection has an association with aggressive behaviors, specifically aggressive behaviors that require contact, then this could increase the change of transmission of a pathogen between the same or different species. This is also true for social behaviors. The three-spined stickleback when infected with a parasite, *Glugea anomala*, were shown to be more social with higher rates of interaction with other fish. This in turn could lead to increases in disease transmission between sticklebacks, thus benefiting the pathogen (Petkova et al. 2018). On the other hand, animal behaviors can also limit disease within a population. Honey bee colonies associated with higher levels of aggression have been shown to more frequently perform grooming behaviors when infected, which potentially decreases disease outcome through cleaning (Carr et al. 2020). The shift in behavior when infected could benefit the pathogen by increasing the disease load and the overall spread of infection within a population, or the shift could hinder the pathogen by increasing behaviors that limit the effects of a disease.

Batrachochytrium dendrobatidis (*Bd*) is a type of fungal infection that has led to drastic decreases in amphibian populations worldwide with the collapse of hundreds of amphibian

species within their environment (Wake & Vredenburg 2008; Cowgill, et al. 2021). *Bd* has an argued point of appearance from anywhere between 1960s-1990s with most of the older 1970s/1980s records being from specimens that were preserved at this time and tested at a later date when the infection became more prevalent (Cowgill, et al. 2021). However, since its appearance it has been attributed to assisting with the sixth mass extinction of the current Holocene epoch (Wake & Vredenburg 2008). Additional factors contributing to the current mass extinction such as climate change and mass habitat fragmentation are correlated with the increase of risks of *Bd* infection and mortality in amphibians (Pounds, et al. 2006). *Bd* infects the skin of the amphibian and continues its life cycle by releasing zoospores from its location either onto the same infected individual or into the surrounding environment (Wake & Vredenburg 2008). The infection is typically characterized by chytridiomycosis or skin lesions on some amphibians (Fisher, et al. 2009). *Bd* is also quite unusual and even more frightening in that it is the first chytrid to infect vertebrates (Wake & Vredenburg 2008). The actual mechanism of death is largely unknown, but is mostly associated with disruptions in oxygen exchange and osmoregulation. The idea is that *Bd* interferes with the general mechanisms of osmoregulation leading to disruptions in circulation and a potential loss of electrolytes overall eventually leading to death (Voyles, et al. 2007).

Anurans, such as frogs and toads, have been the primary focus of *Bd* research with less research in salamanders and far less research in caecilians (Wake & Vredenburg 2008). However, existing research does show potential evidence for the interaction of skin-bacteria on arboreal salamanders affecting behavior. One study while collecting arboreal salamanders for research found that salamanders found in groups were more frequently positive for *Bd* in comparison to salamanders found alone who were more frequently negative for *Bd* (Cowgill, et al. 2021). It is

unclear whether the salamanders were first infected with *Bd* leading them to group together just like the three-spined sticklebacks previously mentioned, or if a few of the salamanders found in the bigger group were more “social” leading to an increased risk of *Bd* transmission. However, this is still an example of how the pathogen-host interaction is related to animal behavior.

Another potential host for *Bd* infection is the red-backed salamander (*Plethodon cinereus*). *P. cinereus* is one type of salamander that is known to clear *Bd* infections without symptoms of chytridiomycosis, unlike other amphibian species with symptoms and higher mortality rates (Becker & Harris 2010). Instead, *P. cinereus* typically experiences a drop in body mass which is common to infections, and a behavior known as limb-lifting in which *P. cinereus* will lift one of four limbs off the ground for a yet to be determined reason (Becker & Harris 2010). There is a dearth of research on the potential influence of *P. cinereus* behavior on *Bd* outcomes.

OBJECTIVE + HYPOTHESIS

Behavioral Observations:

Overall, the interaction between infection and behavior has been partially studied in other model organisms, but not in *P. cinereus*. Additionally, the direct or indirect influence of infection on behavior is divided on increasing or decreasing one of the five main traits of behavior. As such I will observe the individual behaviors of *P. cinereus* keeping in mind three of the five main personality traits of an animal model (sociability, aggression and exploration) to see if there is a wide range of differences between each individual salamander. I will then examine the existing host-pathogen interaction between *P. cinereus* and *Bd* as measured through changes in behavior. Additionally, I hypothesize that the presence of *Bd* will cause significant differences in

aggressive and social behaviors in comparison to the non-infected control groups. This hypothesis remains non directional due to two potentialities. The energy costs of behavior might be too costly for an infected animal and as such are limited to decrease in behavior with infection, or an increase in behavior as induced as a byproduct of infection.

Agreement and Disagreement in Behavioral Observations:

Theory-laden observation is the idea that almost all or some observations in the lab are influenced by our biases (Lau, et al. 2013). If we expect one behavior to occur, then we will be more focused looking for what proves that that behavior has occurred. Remaining entirely objective can be difficult. An example given by Lau et al. (2013) of theory-laden observation is how an older theory on the intensity of acidity in the stomach led many to discredit the initial discoveries of bacteria in the stomach (Thagard 1999). The researchers in the late 90s were so focused on the acid theory that they ultimately ignored or outright rejected rival theories.

Another major factor for observational research is confounding variables, where a third variable is influencing the relationship between the independent and dependent variables. Confounding variables are dangerous in that they can fully change the results of a study. One study even proposed the idea that confounding variables and biases were the only reason that the J-curve association of alcohol-coronary heart disease exists, even if they ultimately decided that it could not be the only factors for this association to occur (Grønbæk, et al. 2021). The J-curve implies that an increase in alcohol intake is related to an increase in heart disease, but other factors such as previous medical history and genetic factors have a greater influence on coronary heart disease. Ultimately, confounding variables are underreported in research (Munkholm, et al. 2020). One study found that nearly a fourth of 120 observational studies in psychiatry did not mention the possibility of confounding variables, and only two of the total 120 studies mentioned

that their results must be interpreted with some level of caution (Munkholm, et al. 2020). The focus here is primarily on observational studies in which the independent variable cannot be controlled, as such there is less room to also control for these confounding variables.

Additionally, most observational studies are not able to use random assignment thus increasing risks of confounding variables (Pouwels, et al. 2016). However, as some confounding variables can be left unobserved I believe it is still relevant to general observations in experimental research. In addition, one study comparing the methodology of experimental and observational designs showed that both groups misuse and misunderstand confound-limiting techniques like random assignment, blocking, matching and random selection (Imai et al., 2008). This can also lead to results that must be interpreted carefully.

Another method of limiting confounding variables and other biases is the use of blind observers. Some research recommends that when possible nearly everyone who can be blind to the study should be including the participants, the observers, and the data analysts (Karanicolas, et al. 2010). One study even found that in comparison between double blind clinical studies and non-double blind studies, treatment effect sizes would increase by an odds ratio of 17% (Shultz, 1995). This is a direct example of how methodology can influence the outcomes of a study, and affect the interpretation of the study. If authors do not list the limitations of their studies, and readers are unable to pinpoint the potential limitations themselves, then published work could be giving the wrong impression to readers in that area of research.

Another method of measuring reliability in observational data is through inter-rater reliability. Inter-rater reliability is a method to determine the level of agreement and disagreement in measurements between trained observers, and has been used to determine the reliability of observational data (Lummel, et al. 2016; Frew et al., 2019). For instance, studying

animal behavior can be difficult at times. A bite can typically be more easily recorded in comparison to slight movements in the trunk. Independent variables in relation to participants can be measured to find any existing correlations with disagreement between observers (Grossetreuer, et al. 2016). Inter-rater reliability is important in determining if the observational data we are collecting is reliable. Higher levels of agreement in raters can show that these observations can be reliably recorded by multiple people. This does not fully eliminate bias as every rater could be influenced by the same bias, but it does help show if there is any individual biases or even some misunderstanding about operational definitions or other measures.

I purpose that measuring inter-rater reliability could help in finding and examining potential error in our behavioral observations. For instance, data in which the majority of observers disagreed cannot be used to determine the significance of a difference between treatment groups. If the observers cannot determine if there were more aggressive behaviors or less aggressive behaviors in a group, then the results comparing that group with others could be inconclusive if not outright wrong.

OBJECTIVE + HYPOTHESIS

Inter-rater Reliability in Behavioral Observations:

In the process of recording salamander behavior, I will also be recording inter-rater reliability between observers to decide on how much agreement between behavioral observations are recorded, and whether or not certain factors are linked with disagreement between observers. The primary aim of this experiment is to determine if the baseline behavior of non-infected salamanders is significantly different from infected salamanders. If the behavior does differ, does it differ based on the traits of sociability, aggression or exploration? Additionally, whether or not

these measures of behavior can be agreed on by independent observers, and if any specific behaviors can cause a decrease in agreeableness between observers. I also hypothesize that observers of aggressive and social animal behaviors will have high measures of disagreement between themselves.

METHODS

Animal Collection

The animal model selected was the Red-backed salamander (*Plethodon cinereus*). All *P. cinereus* were collected from Robertson Field within Meadville, PA. A total of 20 *P. cinereus* were collected. *P. cinereus* were separated into their own individual labeled circular containers (i.e. ID 1-20; date from collection day) with a wet paper towel substrate covering over half of the bottom of the container (soaked by dipping into DI water; cut into $\frac{1}{6}$ original size), and stored in a temperature regulated lab around 18°C (+/-1.5°C) with a regulated light system on from 8am-5pm and off for all other hours. Each of these containers were cleaned for non-exposed animals by replacing the paper towel once a week during animal feeding sessions. For exposed animals, proper procedure was taken to limit the spread of infection. Animals were fed 20-30 fruit flies coated in vitamin dust weekly. After collection, daily mortality checks were made until the conclusion of the study.

General Mirror Test Procedure

One specimen of *P. cinereus* was removed from their individual containers and placed within a rectangular observational container ~4" x 8" with a 2" x 3". The animal was brought into the adjoining lab room at no more than 22°C and left to accumulate for 10 minutes before

observations. The mirror was placed into the container on one of the small walls after an accumulation period of 10 minutes. To note, the specimen was initially placed in the center of the observational chamber facing toward the mirror wall. Meanwhile, the camera and tripod were set up to monitor the specimen so that the camera would be recording the entire length of the observational chamber. After accumulation, each measurement of behavior was taken and recorded from a 2 minute video for each animal. After the 10 minute accumulation of one specimen was over, a second specimen was set to begin the accumulation process during the observational period of the first and so on until all specimens had been observed. Trash bags were hung from the ceiling of the observational area to block any potential visual interference caused by the researcher while the camera is recording for 2 minutes. In this way, the observer and any other accumulating animals would be out of sight of the salamander during recordings. After the observational period was completed for the salamander, *P. cinereus* were placed back into their respective containers and placed back into the temperature regulated lab area. All measures were repeated for every salamander with a slight staggering between predetermined groups of 8-10 specimens that would remain as the non-exposed control and the 10 specimens that will be exposed (see Method of Infection Inoculation; Figure 1). In this way, the behavior of a control specimen was recorded, then a treatment group, then control and so on to limit potential error. Substrate from each observational chamber was disposed of, but the container itself was not cleaned until the end of the study as each is matched with its respective salamander.

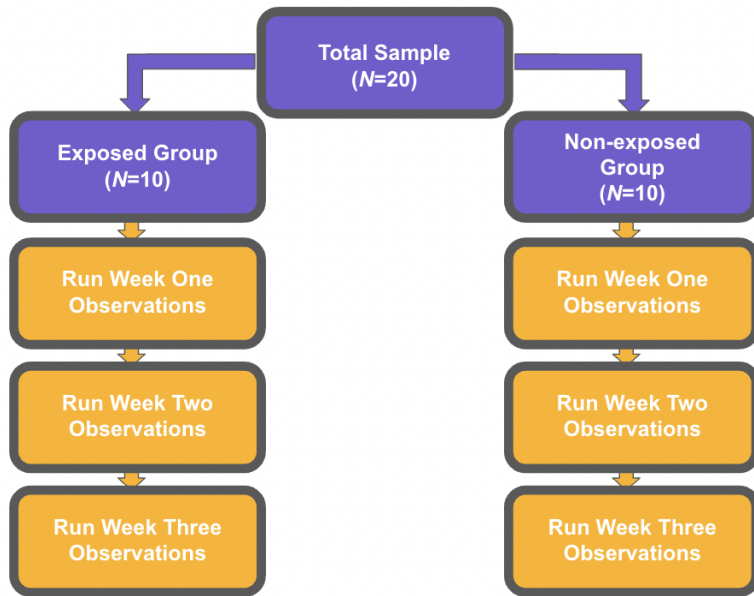


Figure 1: The mixed experimental design showcasing how a total of 20 salamanders were split between exposure groups and were observed over three weeks.

All procedures were a repeat of week one procedures exceptd for additional precautions for the management of *Bd*. To further reduce transmission gloves were used during the cleaning of all substrates. Substrates used with exposed specimens were treated with 10% bleach for 10 minutes. Gloves and all other equipment (including observational chambers and mirror) were properly disposed of within the hazard bin and eventually disinfected and/or treated with 10% bleach, 1% Novaqua® and deionized water between handling of each specimen. The experimental containers were monitored daily for mortality. Procedure would requires any dead amphibians to be contained individually in ethanol or in the freezer. However, there were no instances of mortality in this study. To note, *Bd* is only known to be pathogenic to amphibians, and thus poses no threat to human researchers or other vertebrate animals. Additionally, *Bd* cannot survive for long outside of water or at temperatures above 30°C reducing potential transmission. After the completion of the observational procedure, the exposed salamanders were swabbed for future qPCR analysis with a small sample (2) of the control non-exposed

salamanders being swabbed to confirm no accidental transmission had occurred. Additionally, each individual *P. cinereus* was weighed twice to the nearest thousandths of a gram after swabbing.

Behavior:

In previous literature, the aggressive behaviors of *P. cinereus* have been recorded based on front of trunk raised, all trunk raised, flattened, nose tapping, looking away, looking toward, striking with tongue, and biting (Jaeger, 1984). I recorded aggression into levels with trunk raising with 0 = flattened, 1 = front raised, 2 = all trunk raised. These three behavioral measurements for the trunk have also been previously used when monitoring aggression in response to a predator (Venesky & Anthony 2007). Striking with tongue and biting was recorded as continuous variables. The number of times nose tapping occurred was also counted as a continuous variable. An additional column was made for any unusual or previously unlisted behaviors noted by lab observers. For aggression to be appraised, *P. cinereus* should be incited. This could be done by the presence of either a competitor or predator. In this case, the Mirror Test was used to record levels of aggression in *P. cinereus* as this species fails at visual self-recognition, and thus are unable to recognize their own mirror images. This test has been previously used on sticklebacks and southern red-backed salamanders to measure sociability and aggression based on the continuous measure of behavior and hesitance to approach the mirror (Petkova et al. 2018; Dalton et al. 2020).

Method of Infection Inoculation:

Note that all *P. cinereus* were measured for all baseline behaviors before being exposed. Afterwards, half of the *P. cinereus* sample (10) were then inoculated with *Bd* by a 4mL treatment

of 75,000 zoospores/mL of *Bd* per mL grown in around 1% tryptone broth with an additional 5mL of water to increase spread. This led to a total zoospore count of 300,000 zoospores in the total mixture for each individual animal in the exposed group. The mixture was administered across the skin of the animals in their individual containers by soaking their dorsal sides with the use of a pipette. A bedding change was performed 24 hours after inoculation.

Lab Observers:

A total number of 3 lab observers were tasked with recording *P. cinereus* behavior. All observers have appropriate lab qualifications. I will note that the participants were without supervision and were left to take observations from the videos individually on their own time. This is important as the monitoring of observers can actually affect their observations (Girard & Cohn 2016). Additionally, observers were informed that they should record observations separately and to not make group decisions on behavior for the sake of this study. Two observers were completely blind to the exposure status of the salamanders, thus minimizing bias caused by prior knowledge of treatments. However, the third observer was non-blind to these treatments. Results from both the blind and non-blind observations will be measured together and separately and will be appropriately marked in the results.

DNA Extraction and qPCR Procedures:

After the observational procedure each animal was swabbed along its ventral surface with a sterile cotton-tipped swab, and stored at -20°C for qPCR analysis to detect the pathogen abundance for each specimen.

As per Dr. Venesky's lab procedures for chytrid qPCR, to begin DNA Extraction, the dry bath was turned on to heat DNA samples mixed 40 µL of Prepman Ultra ("P") at 100°C for 10

minutes. After 10 minutes, the swab vials from the dry bath were left to cool for 2 minutes, and then were centrifuged at 13,000 rpm for 3 minutes. All liquid inside the swab vial was added to the pure DNA snap-cap vial. Afterwards, 2.0 μL of the undiluted extracted DNA sample was pipetted into the single dilutions vials with 18.0 μL of sterile, ultrapure reagent water. The vials were centrifuged briefly ($\sim 4,000$ RPM). All vials were placed back into the appropriate freezer box and stored in the freezer to prevent degradation.

A 48 well plate for qPCR was prepared by adding 5 μL of each single dilution into the appropriate well alongside 20 μL of Master Mix. The Master Mix was a combination of reagents and primers such as EM, ED, I Primer, 5 Prime, MGB Probe and Taq. All above reagents were kept on ice while they were not in use. The Master Mix was also kept on ice before and after adding each reagent. One at a time, each frozen reagent was thawed and briefly vortexed.

Afterwards, the zoospore standards (1-6) were retrieved, dethawed and vortexed for approximately 20 seconds. The standards were then added in the appropriate wells with 2 μL of each standard. Finally, 5 μL of ultrapure reagent water was added to the negative control well. The 48-well plate was covered using an adhesive film, making sure that each well was properly sealed. The entire plate was centrifuged using the plate centrifuge for approximately 5–7 seconds before loading into the qPCR machine.

Euthanization:

Upon completion of the experiment, all amphibians were transferred into the care of Professor Matthew D. Venesky for future research and demonstration in the Freshman Seminar Biology class. They will be euthanized in the future with an overdose of MS-222 following protocol (Webb et al., 2005). Individual amphibians will be placed in an individual solution

(~25mL) of MS-222 for 15 minutes and will be confirmed as dead by visual observation of the heart (which is visible through the ventral surface of some amphibians).

STATISTICAL ANALYSIS

Behavioral Observations

To note, language in relation to *Bd* after this point will shift from infection to exposure. I failed to measure pathogen load through qPCR analysis. My standards and subsequent standard curve were correct, and other potential errors in qPCR analysis were accounted for by working jointly with a partner during the procedure.

A few Mixed 2x2 Factorial ANOVAs were conducted in RStudio to determine the influence of time and exposure status on four separate behaviors: nose taps, flat trunks, partially raised trunks, and all trunks raised on both the non-blind observer and blind observers. The counts of tongue strikes and bites were very rarely if never observed in all groups and as such were excluded from analysis. Additionally, week 1 was also excluded due to a video recording mistake that made observing behavior nearly impossible. ID 20 was also removed for the blind observers due to another video error.

RESULTS

Behavioral Observations

Results for the non-blind observer indicated a significant main effect of exposure on nose tap behaviors between exposed including week 2 (2) and week 3 (3), ($M_2 = 15.00$, $SD_2 = 6.60$; $M_3 = 19.33$, $SD_3 = 14.60$) and non-exposed ($M_2 = 5.50$, $SD_2 = 5.19$; $M_3 = 14.70$, $SD_3 = 10.20$)

salamanders, $F(1, 17) = 4.98, p = .026, \eta^2 = .202$. Our effect size indicates that 20.2% of the variance for changes in nose tap behaviors could be explained by the condition of exposure of *Bd*. Additionally, there was a main effect of time on nose tap behaviors between week 2 ($M = 10.00, SD = 7.52$) salamanders, and week 3 ($M = 18.90, SD = 12.35$) salamanders, $F(1, 17) = 4.75, p = .029, \eta^2 = .237$. Our effect size indicates that 23.7% of the variance in changes in nose tap behaviors could be explained by the time between Week 2 and Week 3 spent within the lab and time spent recovering from the exposure treatment. There was no significant interaction effect between exposure and time $F(1, 17) = 0.59, p = .442, \eta^2 = .032$. In the end, there was a general upward trend for nose tap behavior in the non-exposed group of salamanders moving into week 3 (Figure 2).

In comparison, the results for the blind observers indicated a main effect of exposure on nose tap behaviors between exposed ($M_2 = 9.56, SD_2 = 7.63; M_3 = 9.19, SD_3 = 6.26$) salamanders and non-exposed ($M_2 = 2.85, SD_2 = 1.87; M_3 = 7.35, SD_3 = 5.94$) salamanders, $F(1, 16) = 4.54, p = .049, \eta^2 = .221$. Our effect size indicates that 22.1% of the variance for changes in nose tap behaviors could be explained by the condition of exposure of *Bd*. There was no main effect of time on nose tap behaviors between week 2 ($M = 6.03, SD = 6.28$) salamanders and week 3 ($M = 8.17, SD = 5.98$) salamanders, $F(1, 16) = 1.226, p = .285, \eta^2 = .071$. Additionally, there was no significant interaction effect between exposure and time $F(1, 16) = 1.448, p = .246, \eta^2 = .083$.

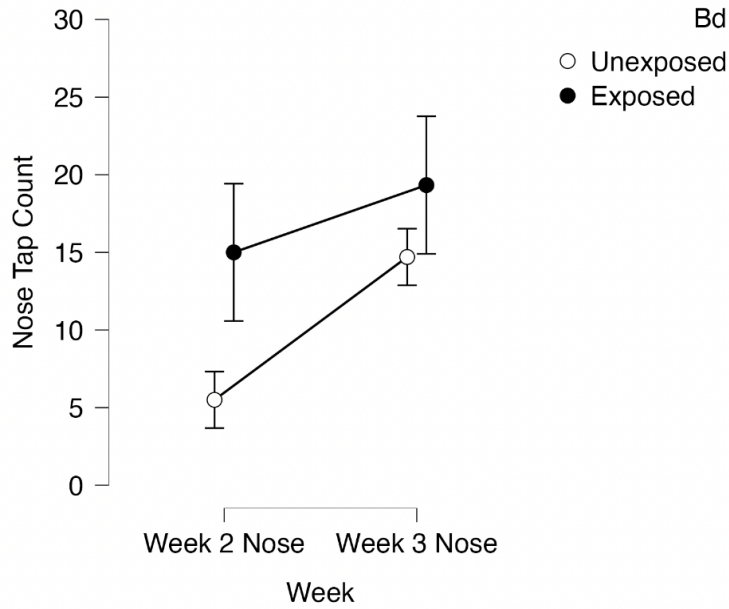


Figure 2: A figure depicting the general trend of the chemosensory behavior of nose tapping vs. the time between week 2 and 3 between the exposed and non-exposed groups. The exposed group generally showed higher levels of aggressive/exploratory behavior between both week 2 and week 3. The non-exposed group showed an increase by week 3 in aggressive/exploratory behavior.

Results for the non-blind observer indicated a main effect of time on flat trunk behaviors between week 2 ($M = .53$, $SD = .61$) salamanders and week 3 ($M = .95$, $SD = .71$) salamanders, $F(1, 17) = 5.39$, $p = .020$, $\eta^2 = .241$. Our effect size indicates that 24.1% of the variance for the changes in flat trunk behaviors could be explained by the time between Week 2 and Week 3 spent within the lab and time spent recovering from the exposure treatment. There was no significant main effect of exposure on flat trunk behavior between exposed ($M_2 = .44$, $SD_2 = .73$; $M_3 = .89$, $SD_3 = .78$) salamanders and non-exposed ($M_2 = .60$, $SD_2 = .52$; $M_3 = 1.00$, $SD_3 = .67$) salamanders, $F(1, 17) = .281$, $p = .596$, $\eta^2 = .016$. There was also no significant interaction effect between time and exposure $F(1, 17) = .015$, $p = .903$, $\eta^2 = .0009$. However, this test has failed

the assumption of normality with a $p < .05$ for the Shapiro-Wilk test. This can be explained by how the behaviors were measured by a count as the number of times the trunk was flattened against the ground of the container, raised and then flattened again. In the end, there was a general upward trend for flat trunk behavior by both unexposed and exposed salamanders moving into week 3 (Figure 3).

In comparison, the results for the blind observers indicated no significant main effect of exposure on flat trunk behaviors between exposed ($M_2 = 1.00$, $SD_2 = .87$; $M_3 = 1.38$, $SD_3 = .79$) salamanders and non-exposed ($M_2 = .95$, $SD_2 = 1.17$; $M_3 = 1.40$, $SD_3 = 1.27$) salamanders, $F(1, 16) = .003$, $p = .960$, $\eta^2 = .00016$. There was also no main effect of time on flat trunk behaviors between week 2 ($M = .97$, $SD = 1.01$) salamanders and week 3 ($M = 1.39$, $SD = 1.05$) salamanders $F(1, 16) = 1.626$, $p = .220$, $\eta^2 = .092$. Additionally, there was no significant interaction effect between exposure and time $F(1, 16) = .00032$, $p = .986$, $\eta^2 = .00002$.

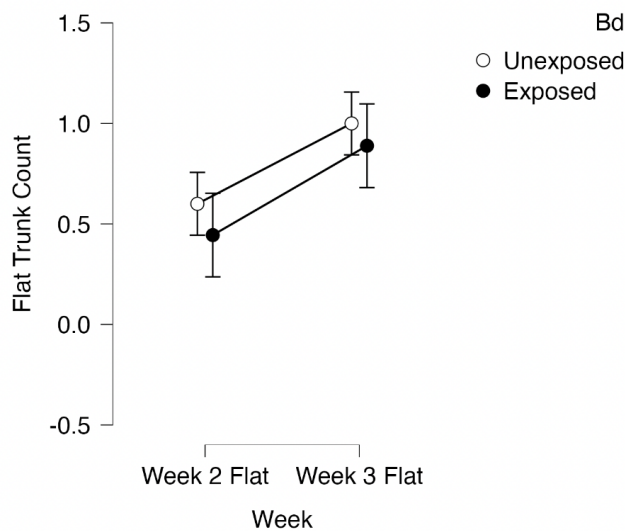


Figure 3: A figure depicting the general trend of the non-aggressive/submissive flat trunk behavior vs. time between week 2 and week 3 between exposed and non-exposed salamanders.

The exposed and non-exposed group both showed a general increase in non-aggressive/submissive behavior by week 3 post-exposure.

Results for the non-blind observer indicated a main effect of exposure on partially raised trunk behaviors between exposed ($M_2 = 1.78, SD_2 = 1.30; M_3 = 1.00, SD_3 = .71$) salamanders and non-exposed ($M_2 = .80, SD_2 = .79; M_3 = .70, SD_3 = .95$) salamanders, $F(1, 17) = 4.21, p = .040, \eta^2 = .216$. Our effect size indicates that 21.6% of the variance for changes in partially raised trunk behaviors is explained by the exposure of *Bd*. However, there was no significant main effect of time on partially raised trunk behaviors between week 2 ($M = 1.26, SD = 1.15$) salamanders and week 3 ($M = .84, SD = .83$) salamanders, $F(1, 17) = 1.83, p = .176, \eta^2 = .096$. Additionally, there was no significant interaction effect between exposure and time $F(1, 17) = 1.18, p = .277, \eta^2 = .059$. However, this test has failed the assumption of normality with a $p < .05$ for the Shapiro-Wilk test. This can be explained by how the behaviors were measured by a count as the number of times the trunk was partially raised, lowered and then raised again. In the end, there was a general downward trend in partial trunk behavior in exposed salamanders moving into week 3 (Figure 4).

In comparison, the results for the blind observers indicated no significant main effect of exposure on partial trunk behaviors between exposed ($M_2 = 2.72, SD_2 = 1.09; M_3 = 3.06, SD_3 = 1.24$) salamanders and non-exposed ($M_2 = 1.95, SD_2 = .96; M_3 = 2.65, SD_3 = 1.03$) salamanders, $F(1, 16) = 2.483, p = .135, \eta^2 = .134$. There was also no main effect of time on flat trunk behaviors between week 2 ($M = 2.32, SD = 1.07$) salamanders and week 3 ($M = 2.83, SD = 1.11$) salamanders, $F(1, 16) = 2.254, p = .153, \eta^2 = .123$. Additionally, there was no significant interaction effect between exposure and time $F(1, 16) = .120, p = .734, \eta^2 = .0007$.

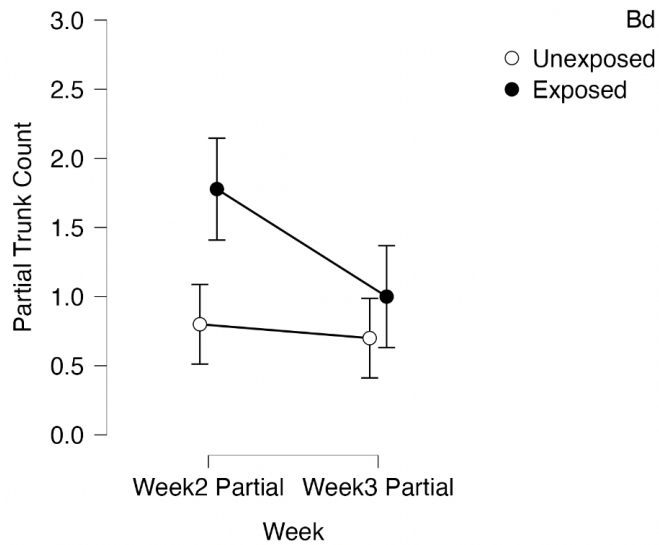


Figure 4: A figure depicting the general trend of neutral partial trunk behavior vs. time between exposed and non-exposed salamanders. The exposed group showed higher neutral behaviors in week 2. However, there was a drop by week 3 post-exposure making both the exposed and non-exposed group perform equal measures of neutral behaviors.

Finally, results for the non-blind observer indicated no statistically significant main effect of exposure on all trunk raised (ATR) behavior between exposed ($M_2 = .78$, $SD_2 = 1.09$; $M_3 = .11$, $SD_3 = .33$) salamanders and non-exposed ($M_2 = .60$, $SD_2 = .52$; $M_3 = .70$, $SD_3 = 1.06$) salamanders, $F(1, 17) = .439$, $p = .508$, $\eta^2 = .025$. Additionally, there was no statistically significant main effect of time on ATR between week 2 ($M = .68$, $SD = .82$) salamanders and week 3 ($M = .42$, $SD = .84$) salamanders, $F(1, 17) = 1.50$, $p = .221$, $\eta^2 = .093$. There was also no significant interaction effect between exposure and time $F(1, 17) = 3.18$, $p = .075$, $\eta^2 = .157$. However, this test has failed the assumption of normality with a $p < .05$ for the Shapiro-Wilk test. This can be explained by how the behaviors were measured by a count as the number of times the trunk was

fully raised, lowered and then raised again. There was a general downward trend in ATR by the exposed salamanders entering into week 3 (Figure 5).

In comparison, the results for the blind observers indicated a marginally significant main effect of exposure on flat trunk behaviors between exposed ($M_2 = 3.11$, $SD_2 = .74$; $M_3 = 3.75$, $SD_3 = 1.69$) salamanders and non-exposed ($M_2 = 2.05$, $SD_2 = 1.14$; $M_3 = 2.95$, $SD_3 = 1.64$) salamanders, $F(1, 16) = 3.976$, $p = .063$, $\eta^2 = .199$. Despite the main effect not being significant this study is underpowered, and as such an increase of sample size could have led to a significant finding. There was also no main effect of time on flat trunk behaviors between week 2 ($M = 2.55$, $SD = 1.09$) salamanders and week 3 ($M = 3.31$, $SD = 1.66$) salamanders, $F(1, 16) = 2.862$, $p = .110$, $\eta^2 = .152$. Additionally, there was no significant interaction effect between exposure and time $F(1, 16) = .093$, $p = .764$, $\eta^2 = .006$.

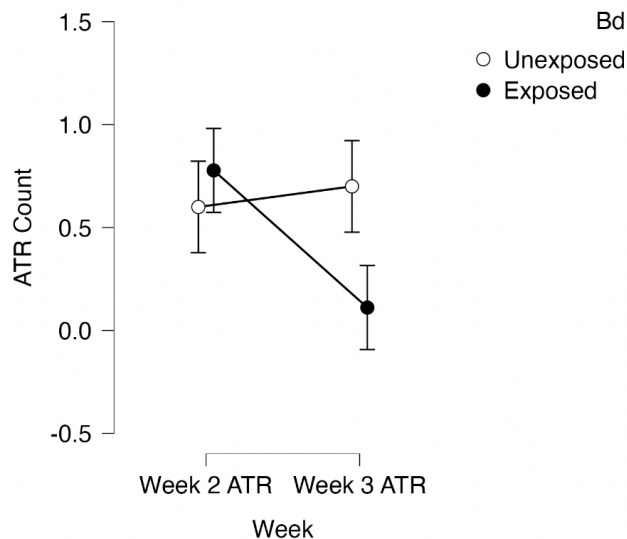


Figure 5: A figure depicting the general trend of aggressive all trunk raised behavior vs. time between exposed and non-exposed salamanders. The exposed and non-exposed group in week 2 have similar levels of aggressive behavior. However, by week 3 post-exposure the exposed group showed a general drop in aggressive behavior.

Masses:

An independent sample t-test was conducted to measure the changes in mass between week 3 and week 2 as influenced by exposure ($M = .028$, $SD = .395$) or non-exposure ($M = -.027$, $SD = .315$) for the salamander sample. This test found no significant effect of exposure and time on mass change, $t(18) = -.344$, $p = .735$, despite exposed salamanders having higher amounts of weight gain and unexposed salamanders having higher amounts of weight loss (Figure 6).

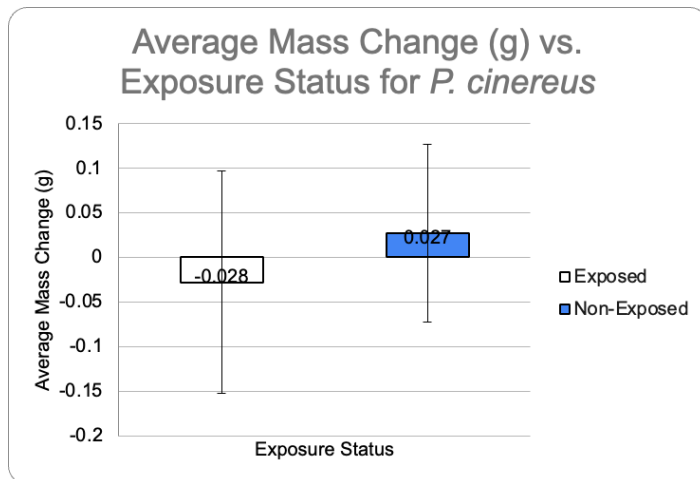


Figure 6: A chart indicating the changes in mass and standard error between exposed ($M = -0.028$, $SD = 0.40$) and non-exposed ($M = 0.027$, $SD = 0.31$) salamanders between week 3 and week 2 of observations within the total sample ($n = 20$).

STATISTICAL ANALYSIS

Inter-rater Reliability (Krippendorff's Alpha):

Inter-rater reliability was measured using Krippendorff's Alpha between three different raters and analyzed using existing conventions. However, results must be interpreted with

caution as one rater was not blind to exposure conditions while the last two were blind. Additionally, when run between only the two blind observers, there were still very similar levels of agreement and disagreement between observers as they were when combined with the non-blind observer.

RESULTS

Inter-rater Reliability (Krippendorff's Alpha):

The rater agreement for measuring nose tap behaviors was the only adequate measure of positive agreement, $\alpha_k = .67$, $SE = .052$. The rater agreement for measuring flat trunk behaviors showed more disagreement, $\alpha_k = .17$, $SE = .067$. The rater agreement for partially raised trunk behaviors showed more disagreement, $\alpha_k = .051$, $SE = .050$. Finally, the rater agreement for ATR behaviors showed significant disagreement greater than disagreement by chance, $\alpha_k = -.112$, $SE = .037$.

DISCUSSION

The primary aim of this study was to interpret the potential behavioral differences between exposed and non-exposed salamanders, and to examine how host-pathogen interactions influence these behaviors. Additionally, this study was made to attempt to measure the level of agreeability between observers of red-backed salamander behavior. Ultimately, there was a significant difference in behaviors with exposure to *Bd*, and raters showed high levels of disagreement with the majority of behaviors measured.

When exposed to *Bd*, *P. cinereus* did see a significant increase in nose tapping behavior in comparison to the non-exposed group. In general, the exposed group had high levels of nose tapping in week 2 and week 3, and the non-exposed group showed an increase in nose tapping by week 3 (Figure 2). Nose tapping for *P. cinereus* and most salamanders is a behavior used to pick up on chemical cues from other salamanders, and from cues within their environment as their chemosensory system is located within their noses (Dalton, 2020). In the case of this study, it can be categorized as an example of bold, exploratory behavior in the presence of a potential competitor. Interestingly, by week 3 the non-exposed group approached similar counts in nose taps with the non-blind observer. Originally, I had assumed that the opposite would occur, in that nose tapping would decrease with exposure and by week 3 the exposed group after presumably fully recovering from exposure, would rejoin with similar nose tap counts as the non-exposed group. I was originally attributing the energetic costs of exposure on behavior as a reason as to why I might see decreases in behavior.

One explanation for why animal behaviors differ in relation to infection could be due to a decrease in available energy. The body focuses on fighting the infection over performing any other behavior. Total energy expenditure is the sum total of energy required to maintain the basic biological functions needed to live and function (Moonen, Beckers & van Zanten, 2021). Total energy expenditure research has been linked to physical activity, diet and metabolism (Moonen, Beckers & van Zanten, 2021). Energy expenditure does influence physical activity; it could also be influencing behavior in general and more specifically traits like exploration (in this regard nose taps), sociability and aggression. In terms of infection, the body must react quickly to survive, and this and the upkeep of immune cells can be energy intensive (Wolowczuk, et al. 2008). If taken from an evolutionary standpoint, the animal must first survive, before they can

pass on their genes. However, this would imply that almost all behaviors should then decrease with infection, when in studies such as with the honey bees their behavior increased with infection (Carr et al. 2020) and with the increase in nose taps that I see in my own results.

One other reason as to why nose taps may have increased could be that exposure to *Bd* itself could have increased the check for chemical cues. Perhaps *P. cinereus* is sensitive enough to even detect the presence of a pathogen and avoid areas containing that pathogen. However, this idea is something of a tin-foil hat idea. This concept would need to be tested for in a future study. However, this would not explain why the non-exposed group saw an increase in nose taps. It could be that time in the lab is what influenced the non-exposed group. Behavioral containers were not cleaned between weeks because they were matched to each individual salamander. Any existing chemical cues within the containers that could be noticed by the salamanders would be their own chemical cues rather than a competitor or neighbor. A familiar chemical cue and the lack of any physical aggression by their mirror image over three weeks could have led to increases in exploration overtime. This idea does partially conflict with a limitation cited by the previous mirror test study on *P. serratus*, in which they attributed high measures of behavior to the fact that the salamander's mirror image is unable to show yielding behavior without the salamander yielding to the mirror. The authors theorized that this could lead to higher viewed measures of aggression (Dalton et al. 2020).

Before I begin to discuss trunk behaviors, I must state that these results must be interpreted carefully. Unlike nose tap behavior, all three trunk measures were very limited in the number of times they actually occurred and standard deviations were very high in comparison to their means. These results could be experiencing a floor effect and as such have led to a skew in their distributions. Additionally, all three measures failed to reach any level of agreeability in

inter-rater reliability with all trunk raised behavior showing disagreement between observers beyond chance. As such, all observers were unable to agree on the frequency of flat, partial and all trunk behavior in *P. cinereus*. Generally, when something like this occurs, observers would be recalled, and asked to re-examine behaviors again. However, I lacked the time and resources to do this.

Finally, blind versus non-blind observations had differing results with only significant differences in non-blind observation, and no significant findings in blind observations. The non-blind observer could have been influenced by prior existing knowledge on the observation of salamanders being greater than the recruited blind observers, or my own biases and desire could have also irreparably influenced the results. Ultimately, process and procedure in the observation section of my comp has led to a divide in my results. The other factor that could have influenced inter-rater reliability is the fact that even when the blind observer was removed, there were still similar levels of agreement and disagreement between them. The familiarity with *P. cinereus*, previous work with the salamanders, and a provided sample sheet with the descriptions of behavior could have been inadequate for observing these behaviors. Typically, observers are trained prior to running inter-rater reliability. This could be that this material is unreliable for future observation studies, and that one future study could be one measuring inter-rater reliability on animal behavior based on changes in the level of training they receive prior to observation. In comparison, one study found that generally there was very little bias in measuring movement and ATR in red-backed salamanders when the observer was blind or non-blind to the sex of the salamander (Marsh & Hanlon, 2004). However they did find some subtle bias where male observers typically observed higher frequencies of behavior regardless of salamander sex, and that both male and female observers typically observed higher frequencies

of aggressive behaviors when only made aware of sex (Marsh & Hanlon, 2004). This could be important to consider for current and future observational research.

When exposed to *Bd*, there was a general increase in both the exposed and non-exposed groups in flat trunk behavior (Figure 3). Flat trunk behavior is a submissive, non-aggressive or timid behavior (Horne, 1998). These findings almost immediately contradict the previous explanation for nose taps. As previously stated, a familiar chemical cue and the lack of any physical aggression by their mirror image over three weeks could have led to increases in exploration overtime. However, an increase in flat trunks would lead to a decrease in exploration and a decrease in aggressive behavior. Another potential explanation could be related to the fact that the increase in flat trunk behavior did not occur until week 3. It could be that the energy that was spent during week 2 to overcome the consequences of exposure led to less available energy by week 3 for the exposed group to pull from. Pathogen response by a host during exposure has been associated with decreased growth rates and increased mortality in amphibians (Garner, et al. 2009). This study was primarily focused on the influence of exposure of *Bd* on frogs during metamorphosis (Garner, et al. 2009). It is possible that these consequences occurred because too much energy was required to combat exposure, leaving too little for metamorphosis. In this study, the salamanders were unable to substitute energetic costs with an increased diet as their diet was kept equal between both the exposed and non-exposed groups. However, the salamanders were not influenced by any other factors such as an energy-costly growth influencing their behavior, as such this study focused specifically on the influence of exposure.

When exposed to *Bd*, there was a high partial trunk behavior in week 2 that decreased by week 3 (Figure 4). Partial trunk behavior is a more neutral behavior mostly unrelated to exploration and aggression as its the normal resting and foraging position (Horne, 1998). As such

it's interesting to see that the exposed group had an increase in partial trunk behavior before matching similar counts of partial trunk behavior as the non-exposed group in week 3. Partial trunk behavior may have occurred more frequently due to a difference in energetic costs. As previously mentioned, TEE is related to physical activity. A limit in physical activity and an increase in potentially low-energy behaviors could be due to the consequence of exposure. This supports the theory that the energy that was spent during week 2 to overcome the consequences of exposure led to less available energy by week 3.

When exposed to *Bd*, there was a general decrease in all trunk raised (ATR) behavior between week 2 and week 3 (Figure 5). ATR is a highly exploratory, high energy, and semi-aggressive behavior. It is the opposite of flat trunk behavior. It is the equivalent of a salamander puffing themselves up to appear as a rougher, tougher competitor. This also supports the theory that the energy that was spent during week 2 to overcome the consequences of exposure led to less available energy by week 3. The high energy cost of ATR and the small number of ATR measured in week 3 for the exposed group could be further evidence that the exposed group lacked the required energy.

As previously stated, the primary goal of examining infection was changed to examining exposure due to pathogen load results being zero. There are two possible reasons for pathogen load to be zero. The initial zoospore dose was limited and could have been too small to ensure infection, or it could have been too small leading to a shorter infection clear time. The other possibility is that I committed an error in either swabbing or within the DNA extraction procedure leaving me with an inadequate example to measure. As such these results can only be interpreted as exposure, and the potential influence of exposure on behavior. However, this has

happened before and exposure has shown to lead to consequences that can increase mortality in frogs (Garner, et al. 2009).

An additional limitation to this study that has yet to be mentioned is that the previous history of my sample is entirely unknown. Unlike rats bred and raised within the lab, these salamanders were all taken from a wild population. Any factors that might have influenced energy expenditure or behavior like previous infection history or injury were nearly impossible to take into account. For instance, I could have caught a subsample that could have been infected by any form of pathogen a week or day prior with no way to control for this factor. This also prevented me from being able to match between my two groups to limit confounding variables. For instance, if there was an influence on previous history I could have split the two groups more evenly.

A future study to build off this work could be more closely measuring the rate of energy expenditure with infection and behavior. If this idea is true that the consequences of exposure were visible over a week after exposure occurring, then it is important for properly measuring the time of recovery in a population. As previously stated, changes in behavior caused by infection have been shown to increase predation. These changes in competitive and exploratory behaviors could prove to be detrimental in the wild.

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