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THE EFFECTS OF TEMPERATURE ON BATRACHOCHYTRIUM DENDROBATIDIS RESISTANCE AND HEART RATE IN THE POLYMORPHIC EASTERN RED-BACKED SALAMANDERS, PLETHODON CINEREUS

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Chapter 1

Title: An inexpensive and non-invasive method for measuring heart rates in terrestrial salamanders

Introduction - Measuring heart rate has practical uses in many biological studies, it is an indirect indicator of metabolic rate (Bennet 1972; Bradford 1983; Butler et al. 2004) and stress (Sgofio et al. 1997; Korte et al. 1999). Metabolic rate, in turn, is an indicator of whole animal stress in plethodonid salamanders because it increases in response to the release of stress hormones (Wack et al., 2011). Furthermore, ectothermic animals, heart rate increases in response to thermal stress (Taylor et al., 1996). Therefore, heart rate measurements can be used as a substitute for both metabolic chamber studies which measure O_2 consumption or CO_2 production, and environmental stress studies which quantify measures of stress through hormone release.

Traditionally, heart rate measurements are performed through electrocardiography, seismocardiography (Aubret et al., 2013), and a variety of internally implanted data loggers (Altmiras et al. 2000). The methods used are dependent upon the application, sample size and species, and funding. Here I demonstrate a simple and inexpensive method of measuring heart rate in small salamanders (< 2g) in laboratory studies. Using a magnifying glass, test tube, and video recording device, researchers can use this method to estimate heart rate in the field.

Ectotherms are physiologically sensitive to temperature changes in their environment (Calosi et al., 2008). Within a range of temperature around the thermal optimum, organisms are capable of maintaining homeostasis and high physiological performance (Huey and Stevenson, 1979). At extreme temperatures, outside of the optimal range, organisms are unable to regulate

their body temperature and maintain homeostasis. This compromises an organisms' basic physiological functions such as locomotion, growth, immunity and reproduction (Deutch et al., 2008). Individuals adjust their physiology in accordance with changing environmental conditions to maintain performance (Romero et al. 2009; Huey et al., 2012). Endothermic animals are able to regulate their own body temperature, expending large amounts of energy in order to metabolically control their body temperature near their physiological optima (Randall et al., 2002). In contrast, ectotherms have limited capacity to metabolically control body temperature and must rely on environmental temperatures to sustain homeostatic equilibrium. To maintain physiologic performance in non-optimal temperatures, ectotherms must alter their heart rates to promote oxygen delivery to more important physiological functions (Gollock et al., 2006; Franklin et al., 2007). As a result, temperature directly affects heart rate in a predictable manner such that heart rate is positively correlated with temperature (Lillywhite et al., 1999).

The Eastern Red-backed Salamander, *Plethodon cinereus*, is a common and ecologically important terrestrial salamander found throughout the northeastern United States and southeastern Canada (Anthony & Pfingsten, 2013). Plethodontids such as *P. cinereus* are commonly used models for studies of behavioral ecology, community ecology, physiological ecology, and life-history evolution (Bruce et al., 2000; Bonett & Gifford 2016; Jaeger et al., 2016; Woodley 2017). *Plethodon cinereus* is strongly limited by temperature and actively seeks optimal temperatures between 16-18°C. They become thermally incapacitated between 32-33°C (Feder & Pough, 1975). Three studies have investigated the effects of temperature on heart rate (Weitzel & Mueller 1973) and metabolic rate (Moreno, 1989; Petruzzi et al., 2006) of *P. cinereus*. In each study, researchers showed that both metabolic rate and heart rate increase with temperature. Previous studies used internally implanted data loggers to measure heart rate at

different temperatures (Weitzel & Mueller, 1973). Implanted data loggers are expensive, hard to implant in small salamanders (<2g), and can alter physiology and behavior. Additionally, data loggers can alter behavior and increase energetic expenditure (Guillmette et al., 2002; Bridger & Booth, 2003) potentially confounding measured values. Implanting data loggers in small animals like salamanders could induce stress, which can alter heart rate in amphibians (Laming and Austin, 1981; CordeiroDeSousa and Hoffman, 1985; Hillman et al., 1987; Wahlqvist and Campbell, 1988). Thus, finding a new, minimally-invasive method could allow researchers to record accurate heart rates quickly and cheapl for a large number of salamanders. In this study, heart rates of *P. cinereus* were examined at different temperatures using a simple and inexpensive method.

Methods - Heart rates were measured for 64 striped adult *Plethodon cinereus*. All *P. cinereus* were collected (ODNR permit # 92-112) by hand from the Manatoc Boy Scout Camp property (MBSC; 41°13'37.2"N, 81°31'17.2" W) in Summit County, OH. Salamanders were housed in a temperature-controlled room on a natural photoperiod in individual vented plastic containers (13.7 x 11.4 x 6.4 cm) with non-bleached paper towels soaked with 13mL of natural spring water. When not undergoing experimentally controlled temperature treatment, animals were maintained at approximately 16.5°C. For the duration of the experiment and to avoid spatial pseudoreplication (sensu Hurbert 1984) I housed all salamanders in a single cold room at approximately 16.5°C. Salamanders were further separated into a cool treatment and a warm treatment. I used heating pads to raise the temperatures of individual housing chambers in the warm treatment. Salamanders in the warm treatment were maintained on tank heat mats (8 x 18in Zilla© 24 watt) with 5 to 6 animals per heat mat. Because heat mats radiate heat into the surroundings, a gradient of values was produced. Resulting salamander body temperatures

ranged from 20°C - 25.6°C in the warm treatment and from 15.6°C - 19.5°C in the cold treatment. In addition, warm temperatures would often cause moisture in the individual containers to condense on the lid, therefore water was tapped off the lid and back onto to the substrate for all salamanders. Tapping the lid and randomizing salamander positions in each treatment were done daily to prevent spatial pseudoreplication. Salamanders were given 6 days to acclimate to their temperature treatment. On day 6 salamanders' heart rates were recorded using an 8 megapixel Iphone 6 camera mounted to a SZ-PT Olympus dissecting microscope with Gosky smartphone digiscoping adapter for stability. The scope was fitted with an LED universal illuminator with bifuricated fiber optics capable of illuminating the salamander from various angles and to prevent heat transfer from non-LED bulbs. Prior to measuring heart rate, the temperature of salamanders and their individual plastic containers were recorded using a infrared thermometer (Extech, product #42540). To record heart rates, salamanders were chosen at random from either treatment and never directly handled as handling can cause stress in amphibians (Narayan et al. 2012) and stress can affect heart rate in other ectothermic species (Braby and Somero 2006). Instead, individuals were kept in their containers and ushered into a slightly moistened glass test tube (13 x 100mm). Test tubes were acclimated to each treatment temperature prior to use; tubes used for warm treatment salamanders were placed on heat mats and tubes for cold treatment salamanders were stored in the cold room. In addition to acclimating the test tubes, salamanders in the warm treatment were recorded under the dissection scope slightly raised above a smaller heat mat (6 x 8in Zilla© 8 watt) resting on the base of the scope to prevent heat loss from the salamanders. Heart rates of salamanders in the cold treatment were recorded slightly raised off the base of the scope. Once in test tubes, individuals were placed under the microscope and given 1 minute to acclimate. After acclimation, the test tube was very slowly rotated so that the ventral surface of the salamander could be viewed through the scope.

Heart rates could be seen through the ventral surface and video recorded for a continuous 60 seconds. Videos were viewed and analyzed at a later date using common computer video software. Videos were slowed down 1.5x the original speed and heart rates were counted for a consecutive 60 second video. Heart rate videos were viewed and counted 3 separate times in order to be sure of count accuracy.

Validation – Measured heart rates were compared to the only published heart rates for *Plethodon cinereus* (Weitzel & Mueller, 1973). I described an almost identical significant positive correlation (Fig. 1; $R^2 = 0.7083$; $p < 0.001$) between heart rate and salamander temperature. At the range of temperatures tested Weitzel & Mueller reported mean heart rates of about 50 – 105 bpm whereas I reported values of 60 – 110 (Fig. 1). There were further similarities in the slope of each trendline, my method produced a slope of 3.97 while they had a slope of 3.78. Because my heart rate values were measured using a gradient of temperatures, I was unable to compare my values quantitatively to their measured values at precise temperatures. I was only able to compare qualitatively as I did not have the sample size for each precise temperature used by Weitzel & Mueller to make a strong comparison. My heart rate values strongly supported those published by Weitzel & Mueller (1973) however, their study failed to account for body size which affects heart rate in plethodontid salamanders (Feder, 1976). Therefore, I standardized my heart rate values by dividing beats/minute by the mass of the salamander. When standardizing my values, I found a strong positive correlation between heart rate and salamander temperature (Fig. 2; $R^2 = 0.5998$; $p < 0.001$). After standardizing my heart rate values, my measured heart rates across temperatures remained similar to published values of Weitzel & Mueller.

Applications – Possible applications of this non-invasive method to record heart rates for small amphibians are broad and could be used to help understand how amphibians and reptiles are able to manage their body temperature through physiological and ecological tradeoffs (reviewed in Cooke et al., 2004). Reptiles exposed to high body temperatures are able to regulate their heart rate to prevent overheating and extend activity into very hot environments (Cooke et al., 2004). Heart rate telemetry has recently been applied to disturbance ecology in order to quantify stress responses of frogs and lizards to human-induced stressors (Cabanc and Cabanc, 2000). Gentle handling produced tachycardia in lizards (Cabanc and Cabanc, 2000), turtles (Cabanc and Bernieri, 2000), and snakes (Heatwole et al., 1979). However, frogs displayed no tachycardia in response to gentle handling (Cabanc and Cabanc, 2000) because amphibians are able to modulate their heart rate reflexively via their vagi nerves and catecholaminergic responses (Krol and Poliak, 1979; Burggren et al., 1992). However, this may not be true for all stressors, as tachycardia in amphibians has been seen in response to intense exercise (Wahlqvist and Campbell, 1988; Hillman et al., 1987) and predator stimulus (Laming and Austin, 1981; Cordeiro de Sousa and Hoffman, 1985). This method described here could potentially be used to further my understanding of heart rate telemetry in response to physiological and ecological stressors, although some fine tuning may be necessary depending on the model system. For instance, heart rates can be seen through the ventral surface for most amphibians, but some amphibians may be more active and less tolerant of manipulation than *Plethodon cinereus*. Additionally, test tubes may work for most small salamanders, but this method may need to be adjusted to accommodate larger amphibians.

In conclusion, the main advantages of using this method are that the process is quick and easy, affordable, and produces accurate measurements. Using this system in the laboratory

provides researchers with a new and simple method to record heart rates in small amphibians without relying on high maintenance machinery or data loggers. Although this method is most amenable to a laboratory setting, a similar slightly modified process could be used in field locations. This could be done in the field or lab using a mounted magnifying glass and a tripod for a video recording device. However, the clarity and control of light source is most likely superior using the dissecting scope. This method grants researchers a practical tool to gather important ecological telemetry data previously difficult and expensive to acquire.

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Figures

Figure 1.

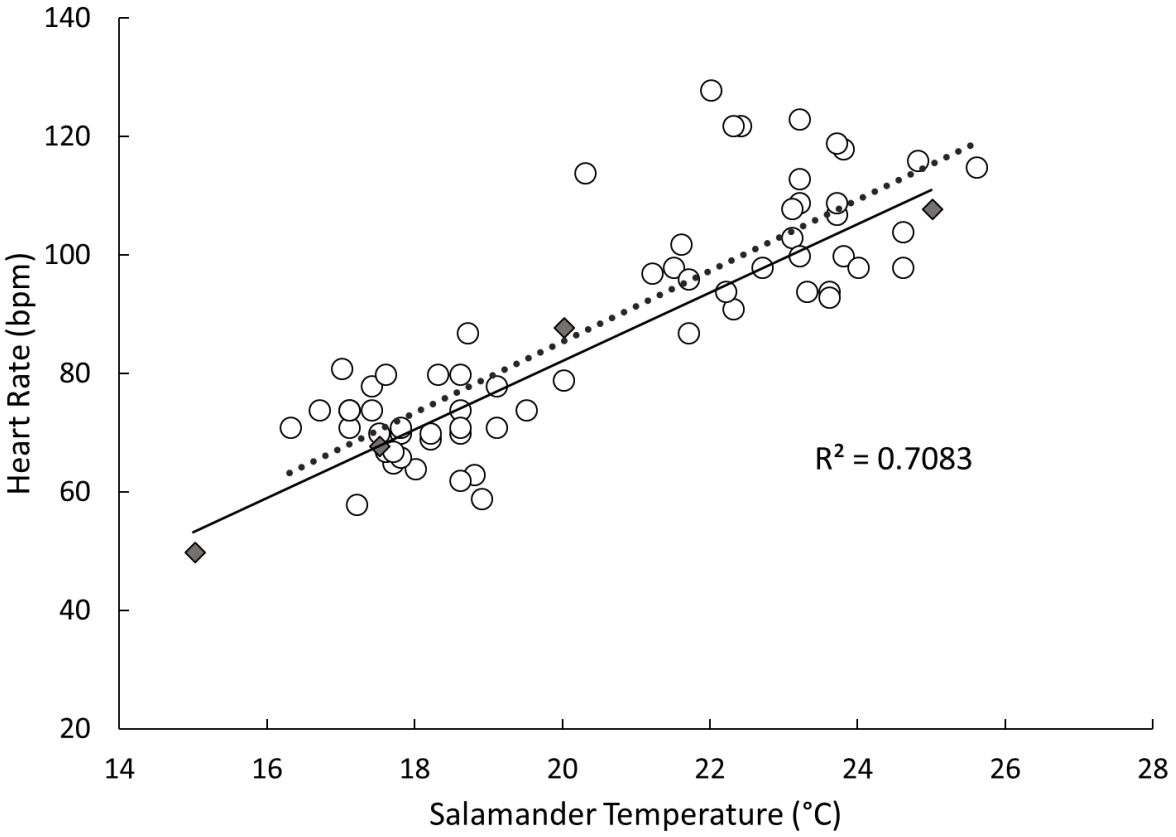

Figure 2.

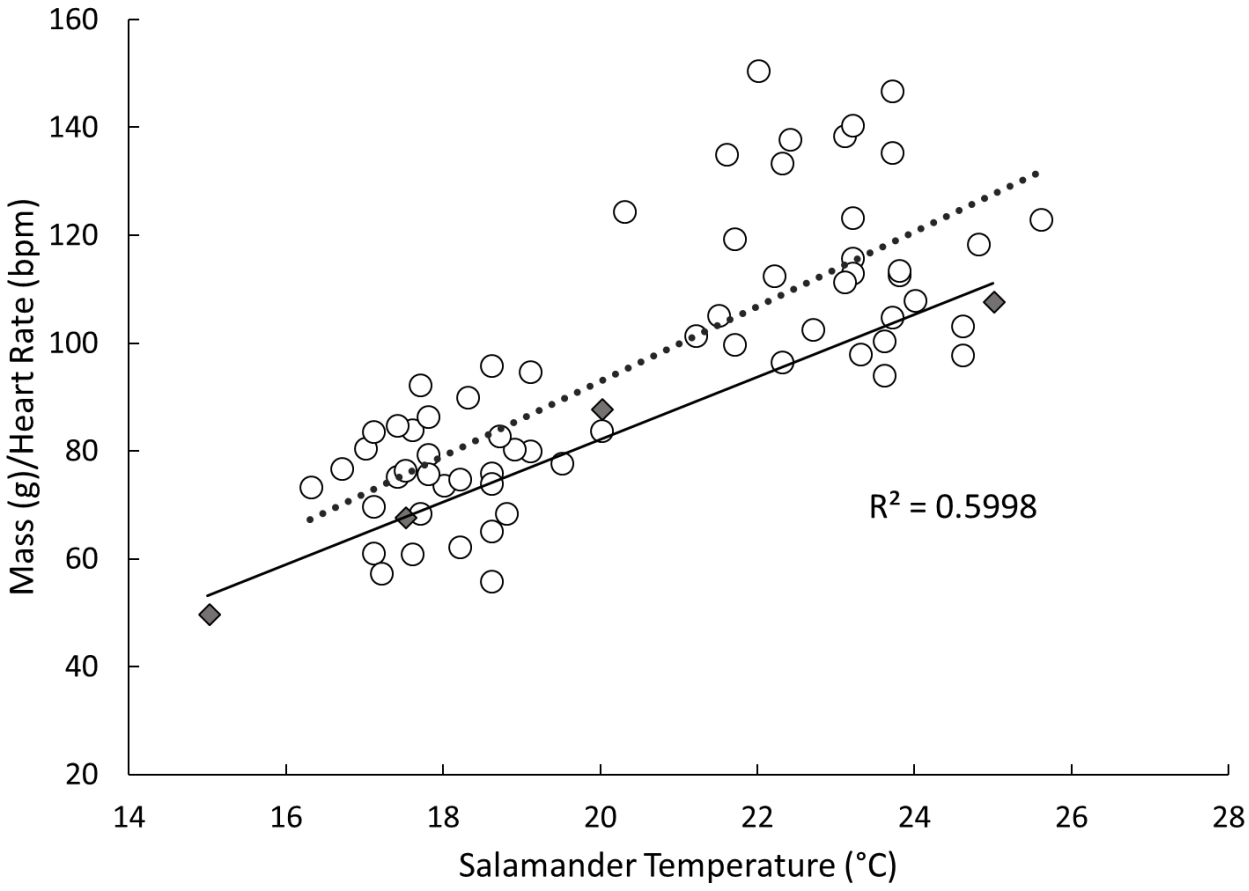

Figure Legends

Figure 1.

Heart rates (beats per minute) were recorded in 64 striped red-backed salamanders *(Plethodon cinereus)* at 'warm' and 'cold' body temperatures. Heart rate significantly increased with body

temperature. Grey diamonds and solid trendline represent heart rates published by Weitzel and Mueller (1973) using implanted data loggers.

Figure 2

Heart rates were recorded in 64 striped red-backed salamanders *(Plethodon cinereus)* at ‘warm’ and ‘cold’ body temperatures. Heart rate was standardized by salamander mass by dividing heart rate beats per minute (bpm) by mass (g). Grey diamonds and solid trendline represent heart rates published by Weitzel and Mueller (1973) using implanted data loggers.

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Chapter 2

Title: Physiological changes in response to temperature and chytrid infection in the color polymorphic Eastern Red-backed Salamander, *Plethodon cinereus*

Abstract

Temperature can change the outcome of host-parasite interactions. However, the way that temperature affects these interactions is not always straightforward because host and parasite species may differ in their optimal temperatures. The amphibian fungal pathogen *Batrachochytrium dendrobatidis (Bd)* is one such parasite whose optimal temperatures for growth (17°C-23°C) commonly differ from those of the hosts. Many amphibian immune responses are thought to be more effective in warmer temperatures, but this may not be universal for cool adapted amphibians because temperatures exceeding an amphibians' optimal range can inhibit immune function. Additionally, *Bd* has a wider breadth of optimal temperatures compared to amphibian hosts, thus, warm temperatures may not always favor amphibian *Bd* resistance. I examined the effects of temperature on *Bd* infection and heart rate of Eastern Red-backed Salamanders (*Plethodon cinereus*) from a color polymorphic population in northeast Ohio. Equal numbers of striped and unstriped salamanders were split into warm or cold temperature gradients and were then challenged with either *Bd* or a sham-exposed control. Striped salamanders at my locality tolerate colder temperatures better relative to the unstriped morph, thus I predicted striped salamanders to have lower *Bd* infections and heart rates at cooler temperatures. I found a nearly significant interaction between morph and temperature on *Bd* abundance: resistance increased across a gradient from cool to warm in unstriped individuals, whereas striped individuals maintained high resistance across temperature gradients. I also found an interaction between the pathogen treatments and temperature on heart rate: heart rates of infected

salamanders were greater at warm temperatures compared to heart rates of non-exposed and non-infected salamanders. Infected salamanders experienced greater percent mass loss than non-infected salamanders which suggests fitness costs associated with resistance regardless of temperature. My results suggest pathogen-induced changes in heart rate and morph specific differences in *Bd* resistance as functions of temperature.

Introduction

Terrestrial and aquatic organisms are facing global population declines, range reductions and extinctions (Barnosky et al., 2011). This decline, termed the 'biodiversity crisis', is arguably part of a sixth major extinction event (Barnosky et al., 2011). Humans play a significant role in this extinction event through habitat destruction, environmental contamination, introduction of invasive species, and global climate change (Hoffman et al., 2010; Vredenburg et al., 2010). Amphibian species and populations are declining more rapidly than birds and mammals (Wake & Vredenburg, 2008; Vredenburg et al., 2010). The rapid decline of amphibians is primarily connected to natural stressors such as competition, predation, resource availability and disease, which are considerably exacerbated by human-induced stresses (Blaustein et al., 2011) and global climate change (Pounds et al., 2006).

Two main hypotheses have been proposed to explain the majority of pathogen induced global amphibian declines (Rachowicz et al., 2005): the climate-linked epidemic hypothesis and the novel pathogen hypothesis. Both hypotheses center around the virulence and transmission of pathogens. Climate change is causing widespread ecological changes (Walther et al., 2002), such as shifts in geographic and elevational distribution (Colwell et al., 2008). The climate-linked epidemic hypothesis proposes that declines are caused by epizootics which are influenced by climate variability (Blaustein et al., 2010) and air temperature (Pounds et al., 2006) caused by

climate change. Research suggests that most pathogens will undergo an increase in range and disease severity as a result of temperature change (Blaustein et al., 2010, Altizer et al., 2013). The novel pathogen hypothesis proposes that the introduction of a new and virulent pathogen to a previously naïve environment leads to widespread transmission and infection culminating in vast devastation to the local populations. The majority of amphibian population declines are likely a result of the combination of these two hypotheses.

The fungus *Batrachochytrium dendrobatidis* (*Bd*) is a new and highly virulent pathogen that is spreading worldwide and killing amphibians (Berger et al., 1998). *Batrachochytrium dendrobatidis,* a water-borne free-living parasite, invades amphibian skin, disrupting electrolyte transfer through it; ultimately leading to death (Fisher et al., 2009; Fisher et al., 2012). Climate change could shift the range of *Bd* through regional temperature alterations, creating new areas that are suitable for *Bd* (Seimon et al., 2007). This expanded range will expose the novel pathogen to the native populations, resulting in an increase in *Bd* transmission and disease severity due to the lack of proper host defenses for mitigating and or defending against transmission and infection.

Recently, a third hypothesis has been suggested to help explain outbreaks of the chytrid fungus. The thermal mismatch hypothesis posits that hosts should be more susceptible to parasites when environmental conditions shift away from their thermal optimum (Cohen et al., 2017). Variable temperatures outside of an organism's thermal optimum will increase the complexity of host-pathogen interactions. This is especially true when considering interactions between *Bd* and amphibian hosts because *Bd* success and amphibians' immune defenses are both highly dependent on temperature (Pounds et al., 2006). *Batrachochytrium dendrobatidis* is associated with longer periods of activity (Voyles et al., 2012) and increasing prevalence of

infection (Woodhams & Alford, 2005) in high elevation and cooler temperatures (Berger et al., 2004, Kriger et al., 2007, Rollins-Smith & Woodhams, 2012). *Bd* has been reported to grow and reproduce best under moist conditions ranging from 17 – 23˚C (Kilpatrick et al., 2010). Temperature also strongly influences immune responses in ectotherms such as amphibians (Feder & Pough, 1975). Many immune functions increase at warm temperatures when metabolic rates are high, and decrease at cold temperatures, when metabolic rates are low and energy is limited (Rollins-Smith & Woodhams, 2012). However, amphibian immune defenses are not universal. Amphibians which have adapted to living in cooler conditions (< 20 ˚C) maintain higher levels of phagocytic activity, antibody production and lymphocyte numbers in cooler temperatures (Plytycz & Jozkowicz, 1994, Maniero & Carey, 1997). The immune defenses of amphibians adapted to warmer environments are thought to be impaired in cooler temperatures (Jozkowicz & Plytcyz, 1998, Lin and Rowlands, 1973, Cone & Marchalonis, 1972). At low temperatures, a number of aspects of immunity in these species are suppressed, including antibody production (Harris et al., 2006, Brucker et al., 2008), lymphocyte numbers (Richards-Zawacki, 2009), and activity of anti-microbial peptides (Geiger et al, 2011). However, amphibian immune defenses are context dependent and are likely most successful at optimal temperatures which individuals are adapted. Therefore, amphibians adapted to cooler temperatures might not experience a reduction in immune activity at cooler temperatures. In order to gain a better understanding of the role that temperature plays in amphibian physiology and in host-pathogen interactions, it is necessary to investigate species with a well understood range of thermal tolerances.

The Eastern Red-backed salamander, *Plethodon cinereus*, is a common terrestrial salamander found throughout eastern North America and southeastern Canada (Anthony &

Pfingsten, 2013). This particular salamander is strongly limited by temperature making it an important species for ecological studies. It actively seeks optimal temperatures between 17-22˚C and becomes thermally incapacitated between 32-33˚C (Feder & Pough, 1975). Thus, this cool adapted species may not experience the same immune costs as warm temperature adapted amphibians. *Plethodon cinereus* is polymorphic, with two common phenotypes: a striped morph with a red dorsal band that extends from the head or neck to the tail (striped), and an unstriped morph that is uniformly black (unstriped). Studies from a locality in which morphs are sympatric suggest that the two color morphs are ecologically differentiated and may respond to differential selection pressures in their shared environments (Anthony et al., 2008). Behaviorally, the two morphs differ in response to disturbance (Fleming et al., 2011), predators (Venesky & Anthony, 2007) and aggressiveness (Reiter et al., 2014). Differences in aggressive and territorial behavior between the two morphs are thought to contribute to differences in prey availability within territories (Anthony et al., 2017), differences in diet (Paluh et al., 2015; Stuzcka et al., 2016), and ultimately access to mates, resulting in assortative mating by color morph (Anthony et al 2008; Acord et al 2013). Physiologically, the two color morphs differ in resistance to *Bd* (Venesky et al., 2015), circulating lymphocyte levels (Davis & Milanovich, 2010) and metabolic rate (Moreno, 1989). Additionally, studies suggest that the unstriped morph is better adapted to drier and warmer conditions (Burger 1935; Test 1952; Williams et al., 1968; Lotter & Scott 1977; Gibbs & Karraker, 2006; Fisher-Reid et al., 2013) whereas the striped morph is found in greater proportions at higher elevations and at northernmost parts of the species range, thus preferring cooler temperatures (Lotter and Scott, 1977; Moreno, 1989; but see Moore & Ouellet, 2015). In colder climates, unstriped morphs have increased mortality (Lotter & Scott, 1977; Moreno 1989) and retreat to underground refugia earlier than striped salamanders (Anthony et al., 2008). Therefore, a polymorphic species, such as *P. cinereus*, with differential temperature preferences

is a good model organism to understand the physiology of an organisms' response to disease under different temperature regimes.

The main purpose of this study was to investigate whether sympatric polymorphic salamanders with known temperature preferences differ in their resistance and their physiological response to *Bd* under different temperature regimes. Unstriped and striped salamanders differ in their resistance to *Bd.* Unstriped salamanders are known to have greater prevalence of infection when not behaviorally avoiding *Bd* compared to striped individuals (Venesky et al., 2015). However, these morphs are predicted to tolerate slightly different thermal breadths which could influence their immune defenses with respect to *Bd* susceptibility and infection. Thus, it is expected that unstriped salamanders will have greater levels of *Bd* infection in cooler temperatures but not in warmer temperatures because the unstriped morph is known to prefer warmer and drier conditions (Moreno, 1989, Anthony et al., 2008, Anthony & Pfingsten, 2013), compared to the striped morph which prefers cooler temperatures (Lotter & Scott, 1977).

In addition to measuring resistance to *Bd,* I also investigated the physiological response to *Bd* infection of *P. cinereus.* I used heart rate as a measure of physiological response to disease (Lowe & Trueman, 1972; Campbell et al., 2007). Heart rate is affected by changes in temperature and possibly *Bd* infection, and in ectotherms heart rate has been used as an indicator of whole animal thermal stress (Helm & Trueman, 1967; Harrison, 1977; Polhill & Dimock, 1996; Braby & Somero, 2006). Thus, salamander heart rates should be lower at each morph's preferred temperature. If both disease and temperature are costly, heart rate is expected to be positively associated with temperature and infection because stress hormones have inhibitory effects on immune system function (Dhabar et al., 1995). Therefore, *Bd* infection should lead to increased heart rate and a decrease in mass. I also predicted generally lower *Bd* abundance at

warmer temperatures because of enhanced amphibian immune systems and suppressed *Bd* growth. In addition, *Bd* infection occurring with temperatures outside of physiological optimum should have compounding effects on heart rate. Unstriped salamanders should maintain lower heart rates and *Bd* infection at warm temperatures compared to striped salamanders. Likewise, striped salamanders should perform better at cooler temperatures and maintain lower heart rate and *Bd* infection compared to the unstriped morph. Furthermore, this study assessed the utility of heart rate as an indicator of thermal and disease-induced stress in a common terrestrial salamander that exists in a range of environmental conditions.

Methods

Collection & Husbandry

All *Plethodon cinereus* were collected (ODNR permit # 92-112) by hand from the Manatoc Boy Scout Camp property (MBSC; 41°13'37.2"N, 81°31'17.2" W) in Summit County, OH on 30 September and 1 October 2016. Salamanders were housed individually with leaf litter in vented glass containers at John Carroll University (University Heights, OH) under a natural photoperiod at 17.0˚C. The *P. cinereus* were fed wingless fruit flies twice per week (approximately 25 *Drosophila. melanogaster* per feeding) with leaf litter bedding changed when needed.

Experimental Design

My experiment utilized a 2 x 2 x 2 fully factorial design investigating the interactive effects of morph (striped/unstriped), temperature (warm/cool), and infection (Bd+/Bd-). One-hundred twenty-three adult *P. cinereus* were used in this study, of which 59 of the individuals were unstriped (27 male, 32 female) and 64 were the striped morph (32 male, 32 female).

Individuals were assigned to one of 8 treatments (n = 15-16/treatment) based on gender and morph such that each treatment had relatively similar numbers of each. On 16 – 22 July 2017, salamanders were separated into 4 temporal blocks (A, B, C, D) with each block separated by a day and moved to vented plastic containers (13.7 x 11.4 x 6.4cm) with non-bleached paper towels soaked with 13mL of natural spring water. For the duration of the experiment and to avoid spatial pseudoreplication (sensu Hurbert 1984) I housed all salamanders in a single cold room at approximately 16.0°C. Salamanders were further separated into two temperature treatments (Cold Xc = 17.4°C; Warm X_w = 22.6°C). I used heating pads to raise the temperatures of individual housing chambers in the warm treatment. Salamanders in the warm treatment were maintained on tank heat mats (8 x 18in Zilla© 24 watt) with 5 to 6 animals per heat mat. Because heat mats radiate heat into the surroundings, a gradient of values was produced. Resulting salamander body temperatures ranged from 20°C - 25.6°C in the warm treatment and from 16.3°C - 19.5°C in the cold treatment. In addition, warm temperatures would often cause moisture in the individual containers to condense on the lid, therefore water was tapped off the lid and back onto to the substrate for all salamanders. Tapping the lid and randomizing salamander positions in each treatment were done daily to prevent spatial pseudoreplication. The salamanders were acclimated to the temperature treatments for 6 days prior to the start of the experiment. The experiment ran for a total of 20 days (16 July – 11 Aug 2017. The first 6 days allowed the salamanders to acclimate to their temperature treatment. The study did not extend more than 14 days after inoculation because *P. cinereus* can clear *Bd* infection in quickly (Venesky et al., 2014). Mass was recorded on day 0, 7, and 14 post-acclimation.

Bd *Exposure*

The *Bd* isolate used in my experiment (JEL 660) was grown in the laboratory in 1% tryptone broth at Allegheny College. All salamanders randomly assigned to the Bd+ treatment, were exposed to a inoculum that contained $1.6x10^6$ *Bd* zoospores per experimental block (A, B, C, D) by direct pipetting onto each salamander. However, salamanders in block B were only exposed to $1.0x10^6$ *Bd* zoospores. Bedding was changed 48h after inoculation to prevent continuous exposure to *Bd* zoospores. Throughout the experiment, all equipment that came into contact with *Bd* or *Bd* infected animals was soaked in 10% bleach to remove any zoospores (Johnson et al., 2003).

Bd *Swabbing*

Bd infection on salamanders was measured by swabbing each exposed salamander on day 7 and day 14 post-exposure. The swab was passed across the dorsal surface of each salamander, including the tail, a total of 15 times. My swabbing protocol followed the published protocol of Hyatt et al. (2007). In brief, a sterile fine-tipped swab (Advantage Bundling; product #MW113) was passed along the dorsal surface of each salamander, including the tail, a total of 15 times. Swabs were stored at 20˚C until further analysis, and the infection burden was assessed via quantitative polymerase chain reaction (qPCR). To prevent cross-contamination with *Bd* or *Bd* DNA, a different pair of Nitrile gloves was worn whenever a salamander or substrate was handled. Throughout this experiment, salamanders were monitored daily for mortality. The number of genome equivalents on each swab was measured using qPCR on a StepOne Real-Time PCR System (Applied Biosystems). The DNA extractions and qPCR analyses followed the methods of Boyle et al. (2004). Test samples were run singly instead of triplicate to control costs (Kriger et al., 2006). I added TaqMan Exogenous Internal Positive Control (Exo IPC) Reagents

(Applied Biosystems) to every reaction well to assess inhibition of the PCR reaction. The Exo IPC system used a standardized concentration of an artificial DNA sequence that was added to each reaction well with its own set of primers and a separate fluorescent probe. The strength of this reaction was used to assess overall reaction inhibition.

I considered infection intensity as the number of *Bd* zoospore equivalents per sample. Zoospore equivalents were calculated by multiplying the genome equivalent values generated by the qPCR assay by 80, which accounted for the 80-fold dilution of DNA from the swabs during extraction and qPCR preparation.

Heart Rate Procedure

On 22-26 July 2017, baseline heart rates were recorded, and blocks were exposed to *Bd* inoculate. Salamanders' container positions were randomized each day to prevent pseudoreplication and all heart rate measurements were taken between 0800 and 1600 hours. Preceding the experiment, salamanders were given 6 days to acclimate to their treatment. Prior to measuring heart rate, the temperature of salamanders and their individual plastic containers were recorded using a infrared thermometer (Extech, product #42540). Salamanders' heart rates were recorded using an 8 megapixel Iphone 6 camera mounted to a SZ-PT Olympus dissecting microscope with Gosky smartphone digiscoping adapter for stability. The scope was fitted with an LED universal illuminator with bifuricated fiber optics capable of illuminating the salamander from various angles and to prevent heat transfer from non-LED bulbs. To record heart rates, salamanders were chosen at random within each block from either treatment and never directly handled: handling can cause stress in amphibians (Narayan et al. 2012) and stress affects heart rate in amphibians (Laming and Austin, 1981; Cordeiro de Sousa and Hoffman, 1985; Hillman et al., 1987; Wahlqvist and Campbell, 1988). Instead, individuals were kept in their containers and

ushered into slightly moistened glass test tubes (13 x 100mm). Test tubes were acclimated to each temperature treatment. Test tubes used for warm treatment salamanders were kept on the same heat mats as the salamanders. In addition to acclimating the test tubes, salamanders in the warm treatment were recorded under the dissection scope slightly raised above a smaller heat mat (6 x 8in 8W Zilla©) resting on the base of the scope to prevent heat loss from the salamanders. While heart rates of salamanders in the cold treatment were taken slightly raised off the base of the scope. This method was validated, and heart rates were shown to be repeatable (DeMarchi, 2016 unpublished data). Once in test tubes, individuals were placed under the microscope and given 1 minute to acclimate. After acclimations, the test tube was very slowly rotated so that the ventral surface of the salamander could be viewed by the scope. Heart rates could be seen through the ventral surface and recorded for a continuous 60 sec video. This procedure was repeated for each group on day 7 and 14 respectively. Videos were viewed and analyzed at a later date using common computer video software. Videos were slowed down 1.5x the original speed and heart rates were counted for a consecutive 60 second video. Heart rate videos were viewed and counted 3 separate times in order to be sure of count accuracy.

Statistical Analyses

I used the "lm" function in R statistical software (R, 2017) to conduct a linear model to test whether the two temperature treatments (cold, warm) significantly affected the recorded salamander temperature on Day 7 and Day 14. Statistical significance ($p < 0.05$) was assessed using the "Anova" function in the "car" package in R.

There are three parameters frequently used to quantify parasite infection: prevalence, infection intensity, and infection abundance. Upon exposure to a parasite, individual hosts will become either infected or remain non-infected (and the percentage of hosts exposed to a parasite

that get infected is termed “prevalence”). Hosts that get infected carry a parasite burden (termed “infection intensity”). Infection abundance unifies the parameters of prevalence and infection intensity because it measures the number of parasites found in all hosts that were pathogen exposed, including the zero values of the hosts that were exposed to but not infected with a parasite. Because plethodontid salamanders are relatively resistant to *Bd* (Fonner et al., 2017; Hess et al., 2015), many of my salamanders had an infection value of “0” and thus I had limited statistical power to analyze infection intensity. As such, I analyzed *Bd* abundance (i.e., the number of *Bd* zoospore equivalents of the salamanders exposed to *Bd*, including those whose value was “0”) using a zero-inflated negative binomial statistical model (using the “glmmADMB” function in the “glmmADMB” package in R). This statistical model considers the response variable a function of a binomial process (uninfected vs. infected) and a count process (negative-binomial distributed infection intensity), consistent with the metric of infection abundance. I trimmed the non-exposed salamanders from the dataset and conducted separate statistical analyses on the Day 7 and Day 14 swabs. In each statistical model, I tested for the main effects of the categorical predictors color (striped, unstriped), sex (female, male) and experimental block (A, B, C, or D) on *Bd* abundance. I also tested for an effect of the salamander temperature recorded on each swab date as a continuous predictor. In each statistical model, I also tested for a color by temperature interaction. Statistical significance ($p < 0.05$) was assessed using the “Anova” function in the “car” package in R.

I used the “lm” function in R statistical software (R, 2017) and tested for the main and interactive effects of the pathogen treatment (treated as a categorical variable “infected”, “exposed but not infected”, or “non-exposed”), color, and temperature on the physiological indices; % mass loss and heart rate on day 7 and day 14. The pathogen treatment was split into 3 categorical variables: infected salamanders were categorized as “infected,” salamanders that

were exposed to *Bd* inoculate but showed no signs of infection were categorized as "exposed but not infected," and control salamanders non-exposed to inoculate were categorized as "non-exposed". I used a 3-pathogen treatment because not all salamanders exposed to *Bd* isolate became infected. Thus, using this approach I were able to compare the effects of the infection and the effects of pathogen exposure to non-exposed salamanders. Statistical significance ($p < 0.05$) was assessed using the "Anova" function in the "car" package in R.

Results

Bd *abundance* – Temperature was significantly different between treatments on Day 7 ($p < 0.001$) and Day 14 ($p < 0.001$). Temperature significantly affected *Bd* abundance on day 7 (Fig. 1; $p < 0.001$) and day 14 (Fig. 1; $p = 0.009$). *Bd* abundance was much higher in cooler temperatures than in warmer temperatures. I also found a significant interaction between color morph and temperature on *Bd* abundance measured on day 14 post-exposure (Fig. 3; $p = 0.032$) but not day 7. As the measured temperature increased, *Bd* abundance on unstriped salamanders went from high to low whereas striped salamanders maintained a relatively low *Bd* abundance across all measured temperatures. This color by temperature interaction seems to be driven by differences in the frequency of infected striped and unstriped salamanders across the measured temperatures (Fig. 3).

Heart Rate – Temperature significantly increased the heart rate in salamanders on day 0 (Fig. 4; $p < 0.001$) day 7 ($p < 0.001$) and day 14 ($p < 0.001$). Higher heart rates were associated with warmer temperatures. Additionally, a significant morph effect on heart rate was observed only on day 14 ($p = 0.038$). But there was a significant morph x temperature interaction on day 0 (Fig. 4; $p = 0.038$), day 7 ($p = 0.046$) and day 14 ($p = 0.048$). Unstriped salamanders had lower heart rates in warmer temperatures but not in cooler temperatures. Pathogen treatment (non-

exposed, exposed not infected, exposed infected) had significant main and interactive effects on Day 7 but not Day 14. There was a significant interaction between pathogen treatment and salamander temperature on salamander heart rate on Day 7 of the experiment (Fig. 5; $p = 0.014$). As the experimental temperature increased, salamanders infected with *Bd* had a steeper increase in their heart rate compared to the change in heart rate of salamanders from the non-exposed or exposed but not infected treatment groups.

% Mass change –There were significant differences in percent mass loss between infected, exposed but not infected, and non-exposed salamanders on day 7 (Fig. 6; $p < 0.001$) and day 14 ($p < 0.001$). Color morph and temperature did not have a significant effect on % mass loss.

Discussion

Being exposed to temperatures outside of an organism's optimal temperature range induces thermal stress. I hypothesized that *Plethodon cinereus* color morphs would differ in physiological response (i.e. heart rate) to infection and resistance to infection. I also expected unstriped morphs to have higher heart rates in the cooler temperatures and lower heart rates at warmer temperatures because they prefer warmer and drier conditions (Lotter & Scott, 1977; Fisher-Reid et al., 2013) compared to striped salamanders. I further hypothesized that unstriped salamanders would have higher *Bd* infection at cooler temperatures and lower *Bd* infection at warmer temperatures compared to striped salamanders.

As hypothesized, unstriped salamanders had lower heart rates at warmer temperatures compared to striped salamanders (Fig. 4). However, striped salamanders did not show lower heart rates at cooler temperatures compared to unstriped salamanders. This may be because heart

rate is an indirect indicator of metabolic rate (Butler et al., 2004) and unstriped salamanders have lower metabolic rates at a range of temperatures including 15°C (Moreno, 1989). Despite differences in metabolic rate between the morphs, there was no difference in heart rates at cooler temperatures. My findings provide evidence of physiological adaptations to different temperature gradients within this species in the form of heart rate. Morphs that differ in their heart rate might also differ in their net allocatable energy available for growth, reproduction, and fitness. This could constitute another mechanism for the maintenance of color polymorphism. For example, in this specific locality there is assortative pairing by color morph (Anthony et al., 2008) However, Moore & Ouellet (2015) argued that a temperature preference may not exist on a wide geographic basis, and that morph distribution cannot be explained by climatic variables. They found no correlation between climatic variables and morph distribution. Thus, my results may not be indicative of all populations of *P. cinereus.* Although morphs may seem to segregate into different thermal habitat, this may depend more upon the census location and season (Petruzzi et al., 2006). The unstriped morph may not display climatic variation across a wide geographic area, but rather preferentially choose warmer microhabitats within their unique and specific populations.

Bd abundance differed between morphs. As the temperature increased, *Bd* abundance decreased on unstriped salamanders, whereas striped salamanders maintained a relatively low *Bd* abundance across all measured temperatures (Fig. 2). Striped salamanders' lower *Bd* abundance at cooler temperatures suggests greater resistance at temperatures near their thermal optima. Unstriped salamanders showed higher probability of infection at cooler temperatures compared to their preferred warmer temperatures (Fig. 3), showing support for the thermal mismatch hypothesis, which suggests that host species are more susceptible to disease at temperatures

outside of their thermal optima. In a recent study, Nowakowski et al. (2016), suggested that the thermal mismatch hypothesis may be better explained through the gap between upper critical thermal tolerances (CT_{max}) of amphibian hosts and *Bd.* Therefore, a host species with a greater CT_{max} relative to that of *Bd,* is more likely able to achieve body temperatures that are detrimental to pathogen growth without themselves succumbing to thermal stress (Rohr et al., 2013). However, CT_{max} may not be the best predictor for *P. cinereus* resistance because this species is highly tolerant of habitat modification and is considered a habitat generalist, meaning they have broad ecological niches along multiple environmental gradients including temperature and precipitation. In addition, *P. cinereus* are geographically wide ranging and likely have local adaptation of thermal optima and environmental conditions across their range. Therefore, the preferred thermal optima of local amphibian hosts may be the best predictor of host immune success rather than an assumed constant CT_{max} for host species and pathogen.

In physiological studies, measuring heart rate serves as an indirect indicator of both metabolic rate (Bennet 1972; Bradford 1983; Butler et al. 2004) and stress (Sgofio et al. 1997; Korte et al. 1999). When amphibians encounter a stressor, the hypothalamic-pituitary-interrenal (adrenal in other vertebrates, HPI/A) system is activated. The HPI axis controls the circulating levels of glucocorticoids (GC) which can bind to intracellular receptors and influence gene expression. The primary role of GCs is to initiate catabolic processes to increase glucose availability, give rise to escape behavior, enhance learning and memory, and enhance some aspects of the immune system to acute stressors at the cost of reduced allocation of energy to growth, digestion, and reproduction (Sapolsky et al., 2000). However, chronic exposure to stressors and long-term elevation of GCs can be especially taxing on individuals and result in many negative effects including neuron death, muscle wasting, inhibition of growth and

reproduction, and immune suppression (Chrousus, 2009). The primary GC in amphibians responsible for regulation of energy allocation is corticosterone. In the Bd/Amphibian system, *Bd* has been shown to be stressful for amphibians as measured by the release of corticosterone (Kinderman et al., 2012, Peterson et al., 2013, Gabor et al., 2015) and by increases in metabolic rate (Wack et al., 2011). Corticosterone has been implicated in reducing host resistance and immunological defenses during *Bd* infection (Carey et al., 1999; Glaser & Kiecolt-Glaser, 2005).

In a recent study, Fonner et al. (2017) tested this hypothesis in plethodontid salamanders. Researchers treated male *Plethodon shermani* with exogenous corticosterone for 9 days, then exposed individuals to *Bd* zoospores. Those given exogenous corticosterone had higher infection abundance compared to control animals. This suggests that corticosterone influences immune function in plethodontid salamanders and likely has a similar effect on *P. cinereus* resistance to the chytrid fungus. Corticosterone has also been implicated in increasing metabolic rate and oxygen consumption in ectothermic animals. Corticosterone increased oxygen consumption in geckos (Preest and Cree, 2008), fence lizards (Durant et al., 2008), eels (Chan & Woo, 1978), and trout (DeBoeck et al., 2001). More recently, corticosterone has been shown to increase metabolic rate (Wack et al., 2011) and likely heart rates in plethodontid salamanders.

Elevated heart rates in amphibians have also been demonstrated in response to stressful situations such as intense exercise (Wahlqvist and Campbell, 1988; Hillman et al., 1987) and predator stimulus (Laming and Austin, 1981; Cordeiro de Sousa and Hoffman, 1985). Therefore, heart rate measurements can be used as a substitute measure for metabolic rate and stress hormones in environmental stress studies where elevated heart rates in infected salamander is likely indicative of stress. I hypothesized that infected *P. cinereus* would have greater heart rates in response to infection. My results demonstrated that infected salamanders did indeed have an

increased heart rate in response to parasite induced stress (Fig. 5). However, heart rates were not elevated in response to infection at cooler temperatures despite them being optimal temperatures for both host and pathogen. Rather, elevated heart rates occurred in infected salamanders in the warm temperature treatment, outside of the organism optimal temperature but within *Bd's* optimal temperature. These findings further support the thermal mismatch hypothesis.

A reduction in physiological performance due to non-optimal temperatures could also be responsible for elevated heart rates of infected salamanders at warm temperatures. Metabolic rate and water loss are fundamental in balancing physiological performance in the face of thermal and hydric stress (Porter & Gates, 1969). These traits interact due to their shared pathway of gas exchange (e.g., skin, lungs) and dependency upon moist respiratory surface to promote oxygen uptake (Maina, 1998). Water loss and oxygen intake are especially important to amphibians, with uptake of both occurring over the same respiratory surface. Lacking lungs, plethodontid salamanders rely on wet skin for cutaneous gas exchange (Gatz et al. 1975). By maintaining moist skin, salamanders lose water to their environment. Reducing water loss rates might impede the uptake of oxygen required to sustain activities related to energy acquisition (Auer et al., 2015) such as immune function. Ectotherms faced with decreased oxygen consumption can alter their heart rates to promote oxygen delivery to more important physiological functions (Gollock et al., 2006; Franklin et al., 2007). For example, Grigg & Seebacher (1999) demonstrated that physiological control of heart rate prevented overheating in bearded dragons (*Pogona barbata*) by increasing heart activity in hot environments. Salamanders could compensate for reduced oxygen consumption by increasing heart rate to promote gas exchange (Lillywhite et al., 1999; Seebacher et al., 2007) and constant oxygen delivery for other functions. Thus, elevated heart rates in infected salamanders may have been influenced by hydric and thermal stress along with

disease induced stress. However, a recent study by Riddell et al. (2018) found that heart rate did not increase in woodland salamander *Plethodon metcalfi* in response to water loss during thermal acclimation. Individuals did not compensate for hydric stress by increasing heart rate thereby increasing blood flow to the skin to promote oxygen diffusion. Rather individuals preferred to minimize energetic costs of cardiac function and decreasing heart rates, to potentially minimize the consequences of stress (Riddell et al., 2018). Therefore, elevated heart rates observed in infected salamanders in the warm temperature treatment are likely due to infection and non-optimal temperatures rather than hydric stress.

Regardless of temperature, infection still came at a cost to individuals. Infected salamanders had a significantly greater percent mass loss than exposed and non-infected salamanders (Fig. 6). Deploying an immune response and resisting infection is energetically demanding (Sheldon & Verhulst, 1996). Energy losses can be mitigated from the redistribution of resources from other physiological processes to immune system function by increasing feeding activity (Lochmiller & Deerenberg, 2000). For example, organisms with access to high quality food have more effective immune responses compared to low quality food (Diamond & Kingslover, 2011) and that food consumption is positively correlated with immune system activity (Tyler et al., 2006). A recent study on *P. cinereus* demonstrated that *Bd*-infected salamanders attacked and captured more flies than non-infected salamanders limiting the extent of nutritional deficits associated with immune system function (Hess et al., 2015) However, Hess et al., (2015) found no difference in percent mass change between infected and non-infected groups. My results suggest that activating and maintaining an immune response has costs, in the form of mass loss, associated with not only infection but also exposure to *Bd*. Furthermore, the salamanders used in this study faced different temperature regimes and accounted for a larger

sample size which could explain differences in my results compared to those of Hess et al. (2015). *Plethodon cinereus,* like many ectothermic organisms, are generally tolerant of mass change and have been shown to resist significant changes is mass when fasted for 35 days (Milanovich & Maerz, 2013). This resistance to mass loss is an evolutionary advantage because large body size has been shown to increase fitness in this species. Larger *P. cinereus* are more likely to inhabit higher quality habitat (Gabor, 1995), have greater success in defending territory (Mathis, 1990; Townsend et al., 1998), and acquire higher quality mates (Mathis, 1991; Anthony et al., 2008). My results demonstrate a clear fitness cost to *Bd* resistance, despite *P. cinereus* having relatively strong resistance to infection and high metabolic efficiency. Not only did infected salamanders face fitness costs but resistance also came at costs to exposed salamanders to prevent infection (Fig. 6). Thus, exposure to *Bd* alone could potentially influence resistant species negatively via costs of constantly preventing infection.

Individuals of *Plethodon cinereus* are resistant to *Bd* and have been shown to clear infection (Becker & Harris, 2010; Venesky et al., 2015). Field-collected plethodontid salamanders rarely test positive for *Bd* from natural environments (Muletz et al., 2014). While most amphibians experience decreased immune function in non-optimal temperatures (Woodhams et al., 2003), *P. cinereus* was highly resistant to *Bd* infection (Fig. 2 & 3) indicating that their immune systems were not impaired by cooler temperatures. Comparatively, warmer temperatures likely aid immune function as exposed salamanders were more resistant at warmer temperatures. *P. cinereus* and other amphibians use commensal skin bacteria in concert with components of the innate immune system (e.g. antimicrobial peptides) to reduce infection burden from *Bd* and other cutaneous pathogens (Colombo et al., 2015). In *P. cinereus* particularly, Becker and Harris (2010) illustrated the importance of antimicrobial peptides and the innate

immune system by removing the skin microbiota of some salamanders and leaving an intact microbial community on others. In both cases, salamanders cleared the infection, but individuals with no skin microbes cleared *Bd* infection slower and at a greater mass loss. Cutaneous and the innate immune system are important in clearing *Bd* infection not only in *P. cinereus* but also in other amphibians (Bletz et al., 2013) and seem to be enhanced at warmer temperatures. Additionally, the immune systems of *P. cinereus* may have been aided by a form of thermoregulatory behavior, termed 'behavioral fever' (Sherman et al., 1998; Gardner & Thomas, 2002; Richards-Zawacki, 2009) through existing gradients of temperature within treatment containers as a result of heating mats. By altering thermoregulatory behavior to sustain a higher-than-normal body temperature, *P. cinereus* may have been able to better fight infections. This is probably due to direct effects of host temperature on the growth rate and survival of *Bd* as well as effects of temperature on their immune system (Blanford & Thomas, 2000).

Bd is highly virulent and regarded as one of the deadliest pathogens on the planet (Fisher et al., 2012) but not all amphibians are equally susceptible to *Bd* (Searle et al., 2011). My results corroborate recent studies that demonstrated high *Bd* resistance in *P. cinereus* despite optimal growth temperatures for *Bd* (Becker & Harris, 2010; Venesky et al., 2015). *P. cinereus* may have enhanced immune function at warmer temperatures, exhibit thermoregulatory behavior, or a combination of both, evidenced by significantly lower *Bd* abundance at warmer temperatures. Regardless, the immune systems of *P. cinereus* were not impaired at cooler temperatures. Thus, cool adapted species may not face the same immune system costs seen in warm adapted species when exposed to cool temperatures. Within their microhabitats, unstriped salamanders displayed better physiological functioning (i.e. heart rate and *Bd* resistance) at warmer temperatures compared to striped salamanders. *Bd* can be stressful for some amphibians, however, infection

seemed to only be stressful for infected *P. cinereus* in combination with thermal stress. At both optimal and non-optimal temperature ranges, *Bd* infection can still pose threats to *P. cinereus* by inducing fitness costs. Better understanding the interactions between *Bd* and *P. cinereus's* immune function and thermoregulatory behavior could further explain these findings.

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Figures

Figure 1.

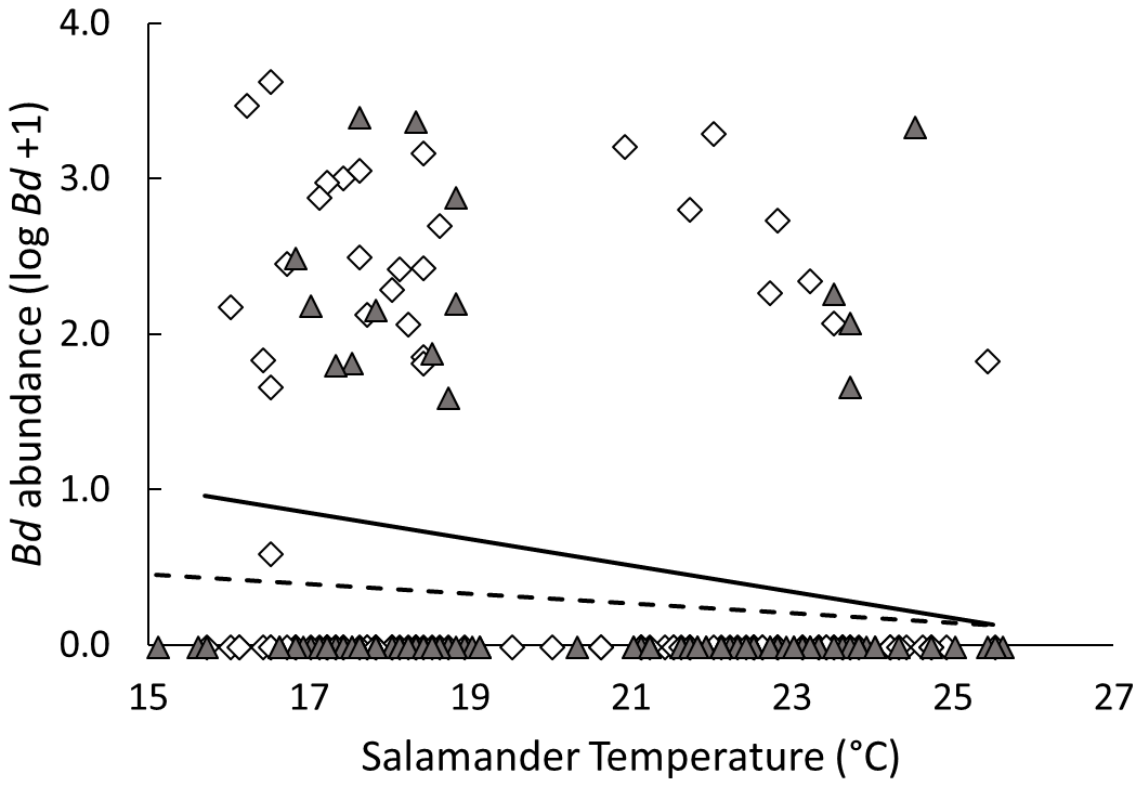

Figure 2.

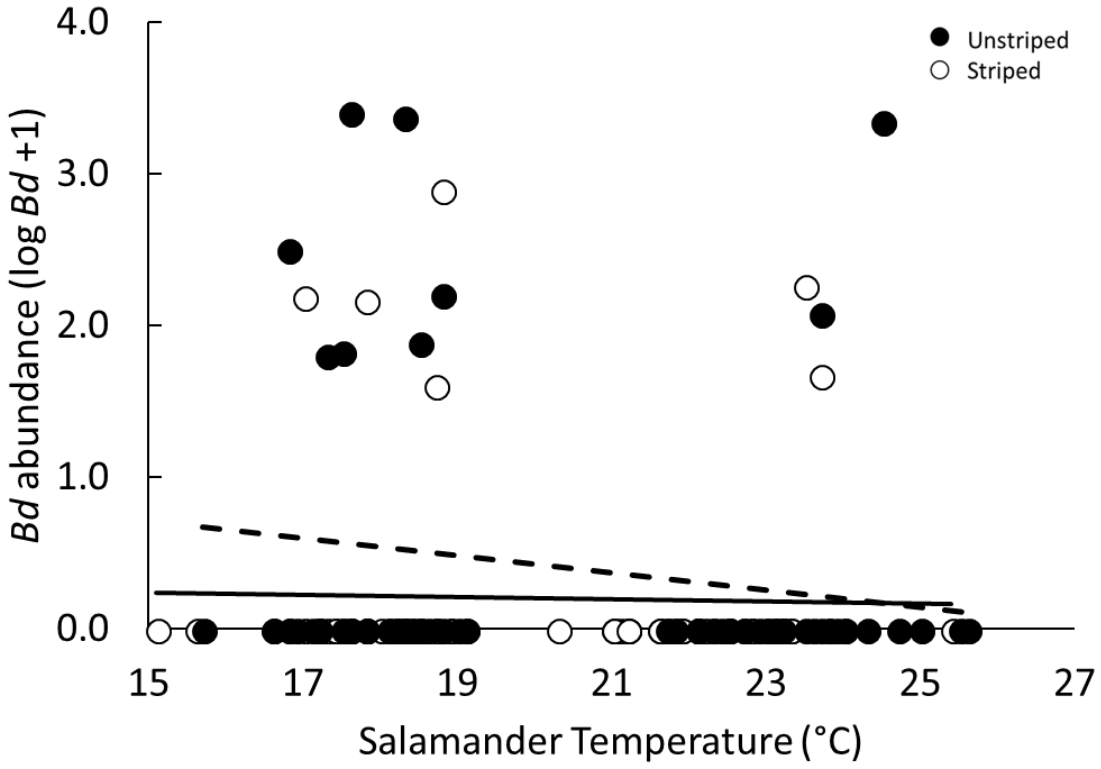
4.0
3.0
2.0
1.0
0.0
Bd abundance (log Bd +1)
15
17
19
21
23
25
27
Salamander Temperature (°C)
Unstriped
Striped

Figure 3.

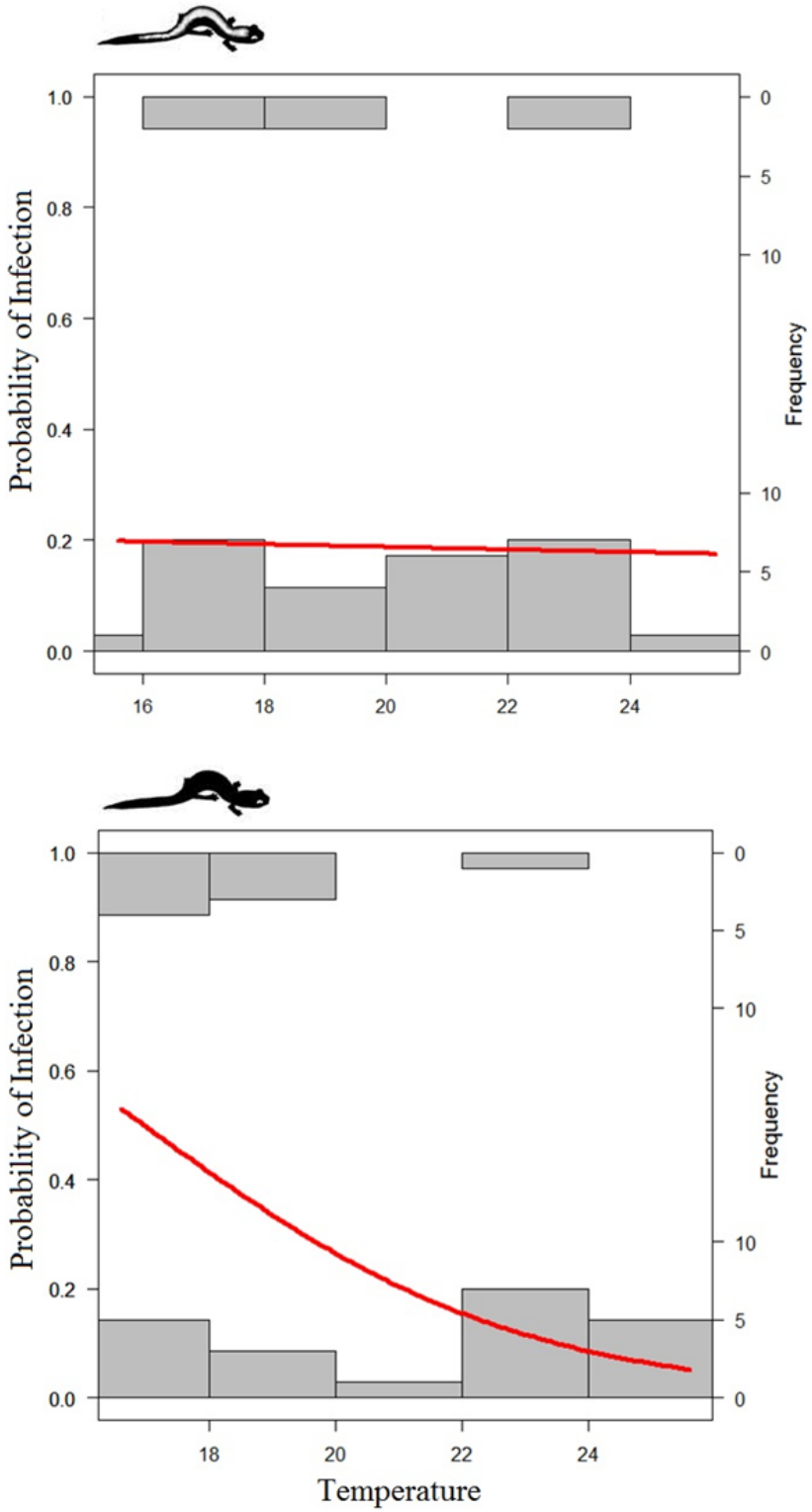
Probability of Infection
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
16
18
20
22
24
Temperature

Figure 4.

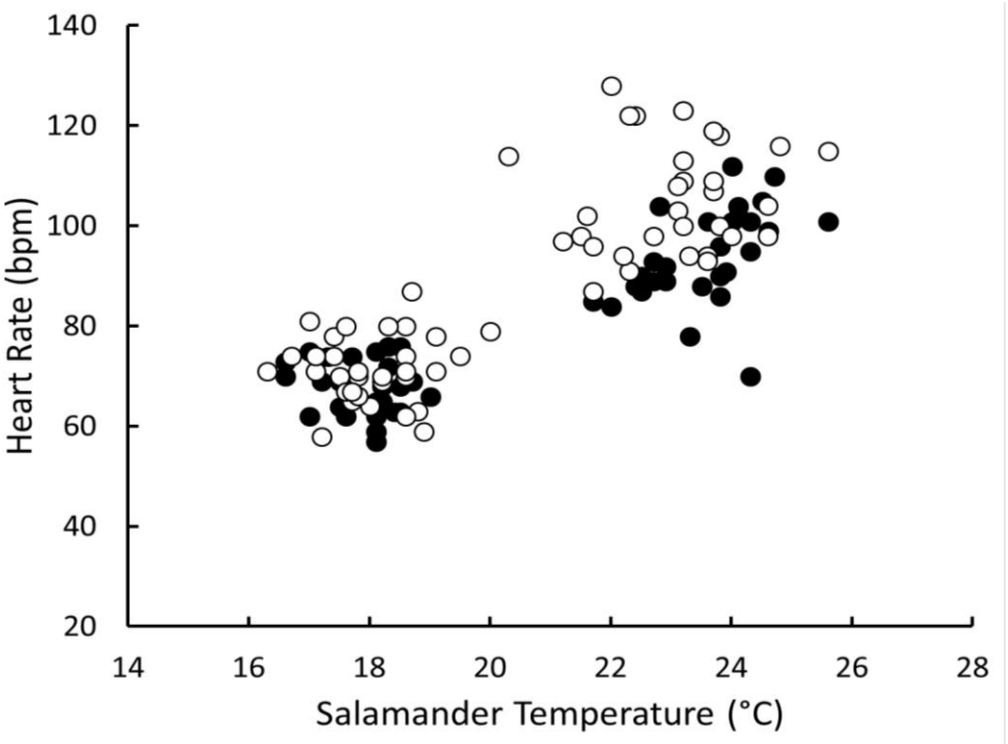

Figure 5.

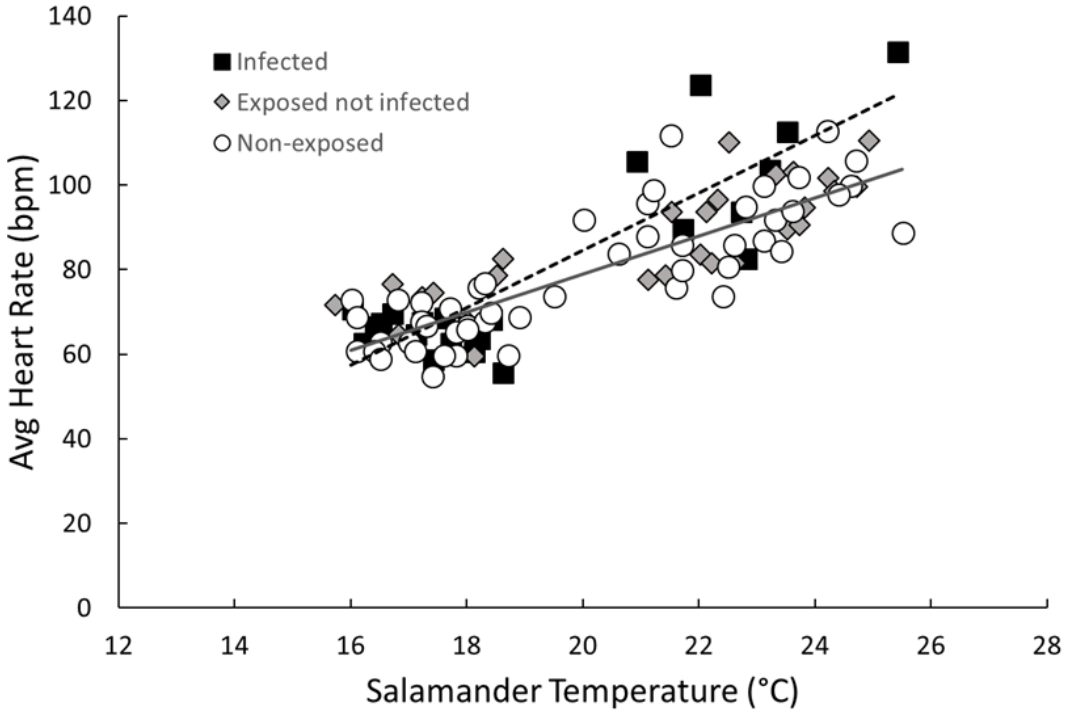

Figure 6.

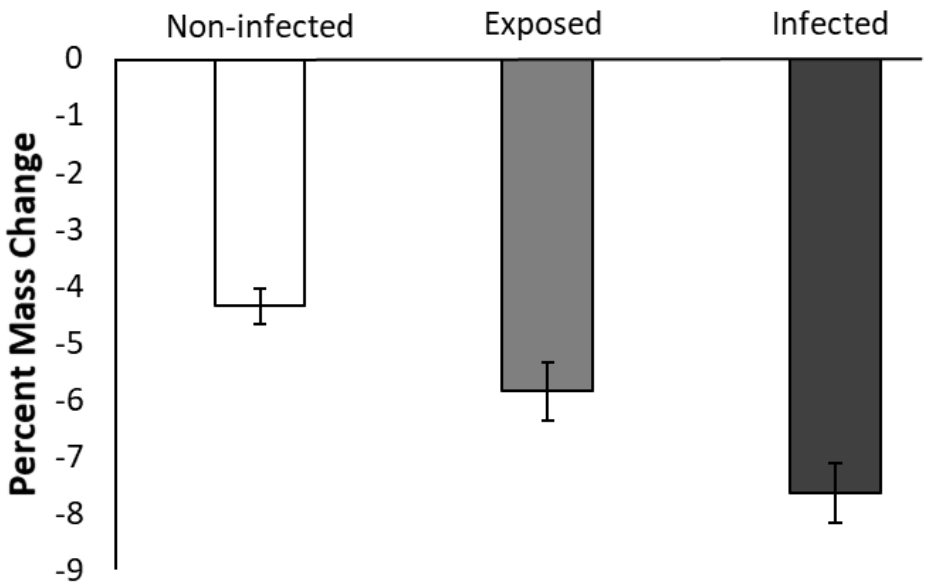

Figure Legends

Figure 1.

Bd abundance (log *Bd* +1) of cold and warm treatments on day 7 (open diamonds) and day 14 (grey triangles). The solid line indicates day 7 trendline and the dashed line represents day 14 trendline. All exposed salamanders were included.

Figure 2.

Day 14 *Bd* abundance (log *Bd* +1) as a function of salamander temperature. Unstriped morphs are represented by open squares and striped morphs by black circles. The dotted line is the best fit line for unstriped salamanders and the uniformly black line represents striped salamanders.

Figure 3.

The probability of infection on day 7 between morphs comparing those exposed to the frequency of infected across temperatures. ‘A’ represents the probability of infection among striped morphs across temperatures. ‘B’ represents the probability of infection among unstriped morphs across temperatures. The red line indicates probability of infection as temperature rises.

Figure 4.

Morph specific baseline heart rates across temperatures. Black circles represent unstriped morph heart rates. Open circles represent striped morph heart rates.

Figure 5.

The relationship between average heart rate (bpm) and salamander temperature (°C) on day 14. Black squares represent infected salamanders. Grey diamonds represent exposed but not infected salamanders and open circles are non-exposed salamanders to *Bd* inoculate. The dotted black line indicates the best fit line of infected salamanders ($R^2 = 0.76$). Both exposed and non-exposed had almost identical trend lines so only one was drawn as seen by the grey solid best fit line ($R^2 = 0.70, 0.69$)

Figure 6.

Percent mass change between infected, exposed but not infected, and non-infected salamanders. White bar represents non-infected salamanders, grey bar represents exposed but not infected salamanders and the dark grey bar represents infected salamanders’ percent mass change. Error bars represent +/- SE.