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Nicholas Britt

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Synthetic viral pyrogen induces behavioral fever in *Plethodon glutinosus* salamanders

Nicholas Britt

Honors Scholars Program
Honors College
Department of Biological Sciences
East Tennessee State University
Johnson City, TN

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Faculty Mentor: Dr. Joseph Bidwell

Faculty Reader: Dr. Rebecca Pyles

Faculty Reader: Dr. Richard Carter

Abstract

Behavioral fever is an essential coping mechanism across ectothermic phyla to aid in combating pathogenic threats. Ectotherms lack internal temperature regulation associated with fever in endotherms; thus, ectotherms can exhibit a behavioral fever response when immunocompromised to thermoregulate by moving to warmer locations. The salamander order Caudata, tend to be keystone species in their resident ecosystems through their role as secondary consumers of invertebrates to maintain the food chain. With growing interest about ecology and conservation of salamanders as species diversity declines, this study was designed to determine if salamanders use their environment to take advantage of behavioral fever. The lungless salamander, *Plethodon glutinosus*, was used to investigate behavioral fever through exposure to the synthetic viral pyrogen polyinosinic:polycytidylic acid (Poly (I:C)) at doses of 15 µg/g by live wet weight. After 24 hours, the induced fever specimens were placed in a linear temperature gradient with a controlled humidity of 95% throughout. Average temperature preferences were then monitored over a 12-hour period and resulted in control animals preferring cold and moderate temperatures between 15-19 °C while all poly (I:C) injected animals preferred higher temperatures from 19-21°C ($p << 0.0001$). This result supports that *P. glutinous* responds to immunocompromising threats such as presented by synthetic viral pyrogen Poly (I:C) through use of behavioral fever.

Introduction

Fever is a common phenomenon experienced by animals as a defensive process upon exposure to pathogenic infection or as response to pyrogenic factors such as protein receptors or genome fragments (Kluger, 1991). Fever is associated with an increase in body temperature to start protective immune pathways, disrupt optimum growth of invading microorganisms, and produce inflammatory processes to combat pathogenic proliferation (Kaiser, 2014 and Urry et al. 2016). Endotherms regulate fever through internal thermoregulation using set-point feedback processes in conjunction with homeostasis. Ectotherms benefit from increased internal body temperatures associated with fever similarly to endotherms but do not regulate body temperature through internal processes relying instead on environmental temperature regulation (Kluger et al. 1975 and Gräns et al. 2012). Ectotherms thus induce fever through movement to warmer locations to raise body temperatures higher than is required for physiological function, this process is called behavioral fever (Casterlin and Reynolds, 1982).

Behavioral fever has been observed in a broad variety of ectotherms including the northern leopard frog, *Rana pipiens*, zebrafish, *Danio rerio*, and ramshorn snails, *Planorbarius corneus* (Gilbertson, et al 2003; Goody, et al 2013; Rakus, et al 2017; Żbikowska, et al 2013). This wide variety of distantly related ectotherms all displaying behavioral fever suggests that the process may have selective advantages that contribute to population fitness (Elliot et al. 2002). This fitness increase may be partially mitigated by the behavioral costs required by individuals to seek out and remain in warmer regions. This change in behavior shows evidence of taxing the ectotherm through reduced foraging times, increased exposure to predation, or a greater need for territoriality encompassing diverse temperature gradients (Parris et al. 2004). The widespread use of behavioral fever also indicates a strong possibility of common phylogenetic origins of the process in ectotherms. This evolutionary origin is supported as ectotherms described as using

behavioral fever commonly utilize the same broad mechanisms involving the hypothalamus and Prostaglandin E to induce fever (Rakus et al. 2017). This retention of behavioral fever across ectotherms indicates the adaptive advantages this fever process provides even with the additional metabolic and behavioral costs required (Rakus et al. 2017).

Although behavioral fever has been described extensively in frogs (Amphibia: Anura), few studies have examined the phenomena in salamanders (Amphibia: Caudata) beyond the Proteidae, Salamandridae, and Ambystomatidae families (Hutchison and Erskine, 1981, Sherman, 2008, and Parris et al. 2004). The phenomena does not seem to be extensively reported in the remaining seven characteristically unique salamander families (Larson and Dimmick, 1993). This discrepancy in knowledge base limits the ability to fully understand how extensively behavioral fever is relied upon in salamanders. The Plethodontidae family is an especially unique family to further investigate for the presence of behavioral fever because this family does not have lungs and relies exclusively on cutaneous respiration. This reliance on cutaneous respiration is associated with changes in the chemical makeup of the skin that creates unique immune protection (Loudon, et al. 2014 and (Woodhams et al. 2015). The species, *Plethodon cinereus,* the red-backed salamander, has been shown to synthesize antifungal metabolites through skin bacterial symbiosis capable of killing *Batrachochytrium dendrobatidis,* one of the leading causes of amphibian chytridiomycosis (Brucker, et al. 2008).

The unique composition of the skin of plethodontid salamanders that allows for cutaneous respiration and an immune protective microbiota creates the potential for manipulation for how behavioral fever is used. The skin may allow for behavioral fever to not be relied on as intensely under immune stress or conversely, these two processes may be used in conjunction with one another for a more effective response (Brucker, et al. 2008; Loudon, et al. 2014; Robak,

et al. 2018). Previous studies in anurans have shown that microbial defense is capable of potentially modulating pathogenic threats as environmental temperatures are relied upon for protection (Robak, et al. 2018, Woodhams, et al. 2015).

The reasoning discussed led to the hypothesis that the Plethodontidae family model specimen *Plethodon glutinosus*, the northern slimy salamander, will display a degree of temperature dependent behavioral fever in response to a chemically induced immune stress to make use of the adaptive advantages fever provides (Hutchison and Erskine, 1981; Rakus, et al. 2017). We hypothesized that *P. glutinosus* would be a good candidate for this behavioral fever study based on similar characteristics to *P. cinereus*. The extent of temperature preference upon exposure to chemical induced stress may indicate the need for these salamanders to access warmer locales for temperature reliant behavioral fever. The chemical induced stress used was the synthesized pyrogen polyinosinic:polycytidylic acid (Poly (I:C)). This chemical has been shown to initiate an immune response through functioning as an immunostimulant that interferes with signaling of B-cells, macrophages, and dendritic cells of the immune system in a holistic innate immune system response (Hason, et al., 2011 and Żbikowska, et al. 2013). This is because the structure of Poly (I:C) is analogous to double stranded RNA found in viruses and has been shown to induce interferon pathways similar to viral infections to directly induce prostaglandins involved in fever and temperature regulation (Koren, et al. 1981; Matsumoto and Seya, T. 2008; Yaron, et al. 1978). Previous studies with invertebrates, fish, and amphibians also support Poly (I:C)'s merit as an immune response inducer (Boltana et al. 2013 and Gan, et al. 2017).

Materials and Methods

Blood Assay Preliminary Tests

The chemical pyrogen Poly (I:C) was first investigated to ensure that the pyrogen would disrupt the immune system enough to prove feasible in inducing behavioral fever. The merit of the pyrogen Poly (I:C) as a fever inducer was evaluated through use of a blood oxidative burst assay. This assay surveys innate immune response by observing phagocytic activity in the presence of a pyrogen. This observation is achieved through use of a chemical mixture that produces chemiluminescence proportional to phagocytic degradation of any present pyrogens (Falso et al. 2015). The assay was conducted using goldfish, *Carassius auratus*, blood. *C. auratus* was chosen for the blood assay model because previous respiratory burst response studies had been shown to work on zebrafish, *Danio rerio*, and proved relevant for other ectotherms, so a similar model specimen was used for the burst assay (Boltana et al. 2013; Goody, et al. 2013; Rakus, et al. 2017). In addition, *P. glutinosus* tend to have small quantities of blood when a large volume of blood is required for the assay.

The Poly (I:C) pyrogen's effectiveness at creating an oxidative burst response was the focus on this part of the study. High levels of phagocyte innate immune system activity would indicate the first stages of fever so provides a benchmark test for a Poly (I:C) immune response. Poly (I:C) was mixed with the blood samples along with zymosan A and luminal to create a mixture capable of measuring fever response through the oxidative burst assay. This assay relies on active phagocytes encountering and responding to blood pyrogens to produce hydrogen peroxide as well as hypochlorous acid (HOCl) through the degradative pathway against the pathogen (Goody, et al., 2013). The phagocytic chemical biproducts react with luminol to cause

fluorescence that is directly proportional to response strength (Gilbertson, et al. 2003; Marnila, et al., 1995).

Blood was extracted through euthanasia utilizing MS-222; fish were immediately taken out after death and the head severed behind the operculum (Amanda and Whiteman, 2006). Heparinized capillary tubes were dabbed in the heart region and caudal vein. The blood was then preserved with heparin (10-30 USP/mL blood) after transferring to an Eppendorf microtube. In an additional Eppendorf microtube, Frog Ringers solution (0.116 mM NaCl, 1.2 mM KCl, 1 mM $CaCl_2$, 2.7 mM $NaHCO_3$) and Luminal (0.25M NaOH, 1M Boric Acid, 10mM Luminal) were added together at a 3:1 ratio of Ringers to Luminal and lightly vortexed three times. In a 96 well plate, wells were filled with 100 µL of blood and 54 µL of Ringers Luminal mixture to a total volume of 154µL in each well. 100 µL of Poly (I:C) stocks at 40, 80, 100, 200, 400nM concentrations diluted in saline water were added to separate experimental wells. After Poly (I:C) stock concentrations were added the well plate was left undisturbed for ten minutes. After this time, a 50uL of Zymosan A stock solution (0.123M zymosan A moderately boiled in 7.6pH phosphate buffer) was added totaling 304 µL per well. The well plate was immediately placed in a plate reader using the following settings: fluorescence: Excitation: 485 nm; Emission: 528 nm; Optics Position: top; Sensitivity: 65, with a 5 sec shaking step prior to the read. The plate reader ran for 30 minutes and at 3-minute intervals recorded data.

Salamander Collection and Holding

For the behavioral fever study, *P. glutinosus* were collected from Carter County Tennessee in the Roan Mountain area in early Fall 2020 and transported back to the laboratory immediately after collection. Fifteen adult specimens between 4.00 to 5.00 grams were collected at dusk through use of spotlight and caught by hand in sloping hill regions with large amounts of

soil, leaf litter, and decomposing wood. Upon capture the specimens were placed in a small plastic container with moist sediment, leaf litter, and humidity regulated with damp sphagnum moss. Salamanders were collected in early fall when ambient temperatures were 14-18 °C. The salamanders were transported back to the laboratory and held in a Thermo Scientific 3990FL incubator (Thermo Fisher Scientific Incorporated, Waltham, MA) at 16°C with two weeks to acclimate. Immature salamanders and salamanders that showed signs of lethargy or sickness were released; in addition, one of the fifteen salamanders escaped due to human error.

Behavioral Fever Chamber Study

The fever response study was initiated by intraperitoneal injection of Poly (I:C) or amphibian buffered saline to living salamanders. A total of 14 healthy salamanders were used in the experiment with two groups of specimens. A nine-member experimental group was injected with a 15 µg/g concentration of Poly (I:C) made by dissolving Poly (I:C) salt in sterile amphibian phosphate buffered saline (APBS) at pH 7.2. The remaining five control individuals were injected with APBS at pH 7.2 (Grayfer, et al., 2014). All injected specimens were weighed by wet mass and injected with a total volume of 500µl, 24-hours before sampling. All specimens had a 24-hour resting period after injection to allow time for the fever response to initiate before sampling (Ames et al. 2013).

Evaluation of thermal preference after exposure was conducted using an automated four chambered temperature-controlled arena. Each 120mm x 120mm sub-chamber was interlocked with small passages and had devices to monitor and adjust temperature and humidity. Each chamber also had a load cell for constant measuring of salamander location over time. The four sub-chambers each incrementally increased in temperature at 15, 17, 19, and 21°C with a controlled humidity of 95% throughout each. Specimens were randomly placed within the sub-

chambers and the salamanders were monitored over a 12-hour time span. Load cells recorded

every minute to determine preferred temperature ranges by average time spent in each chamber

(Figure 1). Voltage readings were monitored by a Keithley voltmeter and data was transferred to

a custom program that uses excel plotting to display which box had a change in voltage at each
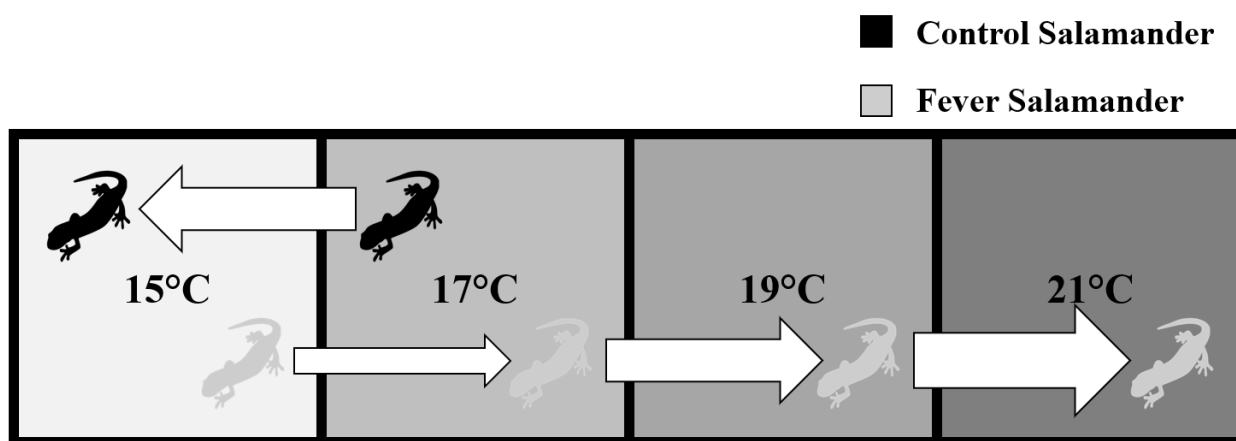
minute recorded.

Figure 1: Temperature chambers used in behavioral fever study regarding *P. glutinosus*. Internal numbers indicate connected temperature arenas in 2°C intervals to form a gradient of temperatures from 15°C to 21°C. The figure presents our hypothesis that control salamanders would prefer an average temperature lower than that preferred by feverish salamanders.

Statistical Analysis

Statistical analyses used Excel Data Analysis Pack with α= 0.05. The blood assay was

tested for significance with a two-factor analysis of variance (ANOVA) without replication

between treatment and time intervals over a thirty-minute period. The behavioral fever chamber

data were examined with a two-tailed equal variance t-test. The behavioral fever sampling took

place over several weeks, so an additional two-way ANOVA test was conducted to examine for

time-based confounding of variables.

Results

The preliminary *C. auratus* blood assay for all blood mixture concentrations with Poly (I:C) resulted in significantly higher fluorescence over controls of blood with Ringers only. In Poly (I:C) experimental groups, the chemiluminescence demonstrated a trend in higher values (CPM) within the first six to nine minutes of the blood assay before dropping off to control levels of fluorescence. A significant difference in chemiluminescence was observed between each chemical treatment group to control groups ($p<<0.0001$) as well as this difference being correlated to the current time interval ($p<<0.0001$). This time difference is relevant in determining the intensity and length of the immune response. The twelve-minute mark appears to be the time interval where experimental and control groups merge (Figure 2).
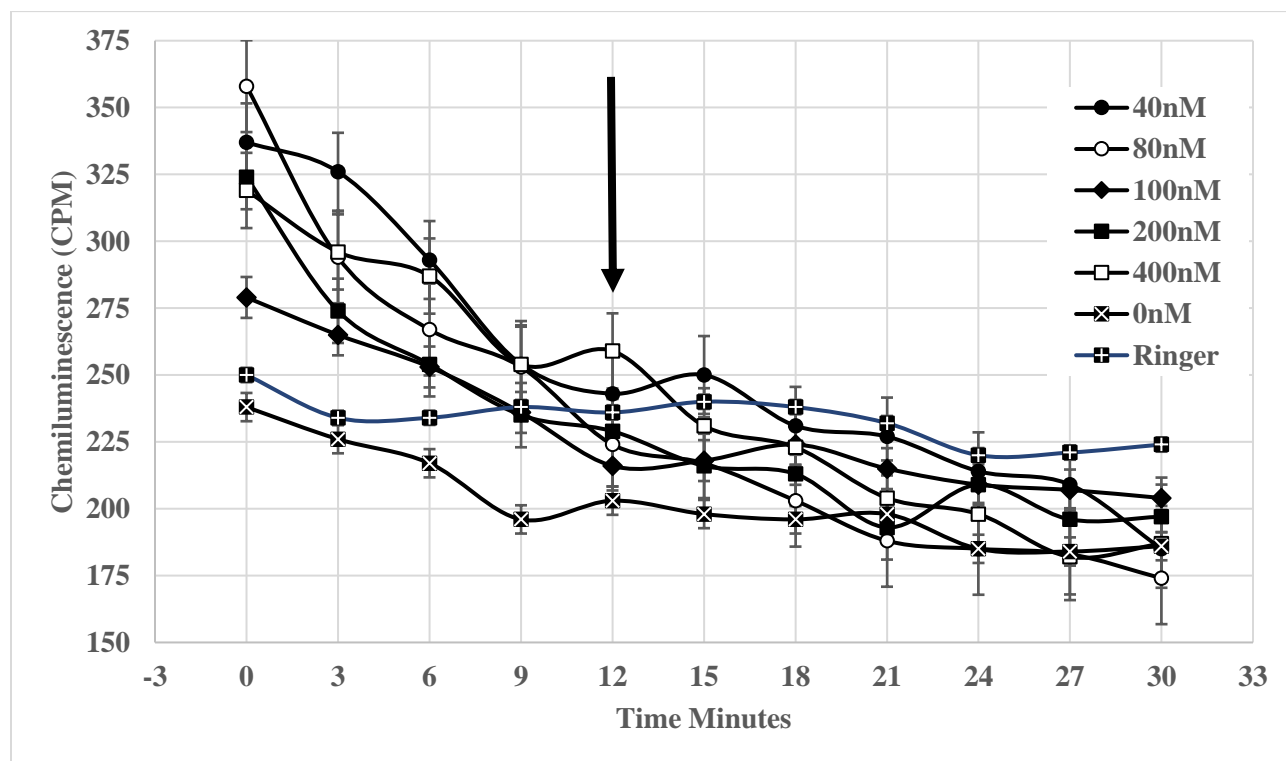

Figure 2: Chemiluminescence of all measured concentrations of Poly (I:C) and controls in counts per minute (CPM). The data were measured by plate reader over a thirty-minute time interval with counts recorded each three minutes. Error bars show one standard error. The arrow shows where experimental samples appear to merge with comparable chemiluminescence values to controls.

The behavioral chamber experiment involving fourteen healthy adult *P. glutinosus* demonstrated significantly different (p<<0.0001) average temperature preferences over the twelve-hour sampling period between experimental (Poly (I:C) injected) and control (APBS only injected) individuals. Control animals exhibited an average preferred temperature range within the cold to more moderate temperatures from 15-19 °C. Poly (I:C) samples preferred moderate to higher average temperature preferences from 19-21°C (Figure 3).
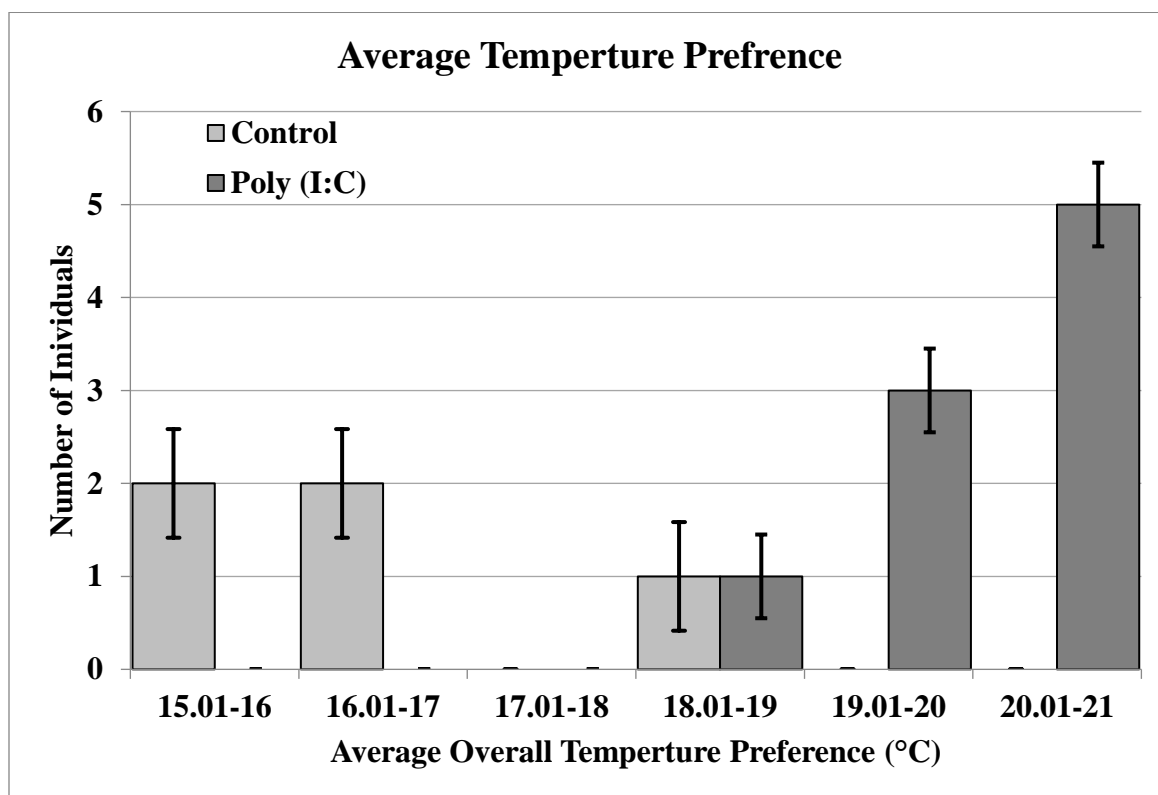

Figure 3: Average temperature preference between control (Ringers injected) and Poly (I:C) injected *P. glutinosus.*. Error bars show one standard error.

The behavioral chamber scan per minute voltage readings revealed average temperature preference in half hour increments throughout the 12-hour sampling period. The average temperature preference deviated between control and experimental groups after the first hour and remained through the rest of the eleven hours remaining in each test run (Figure 4).
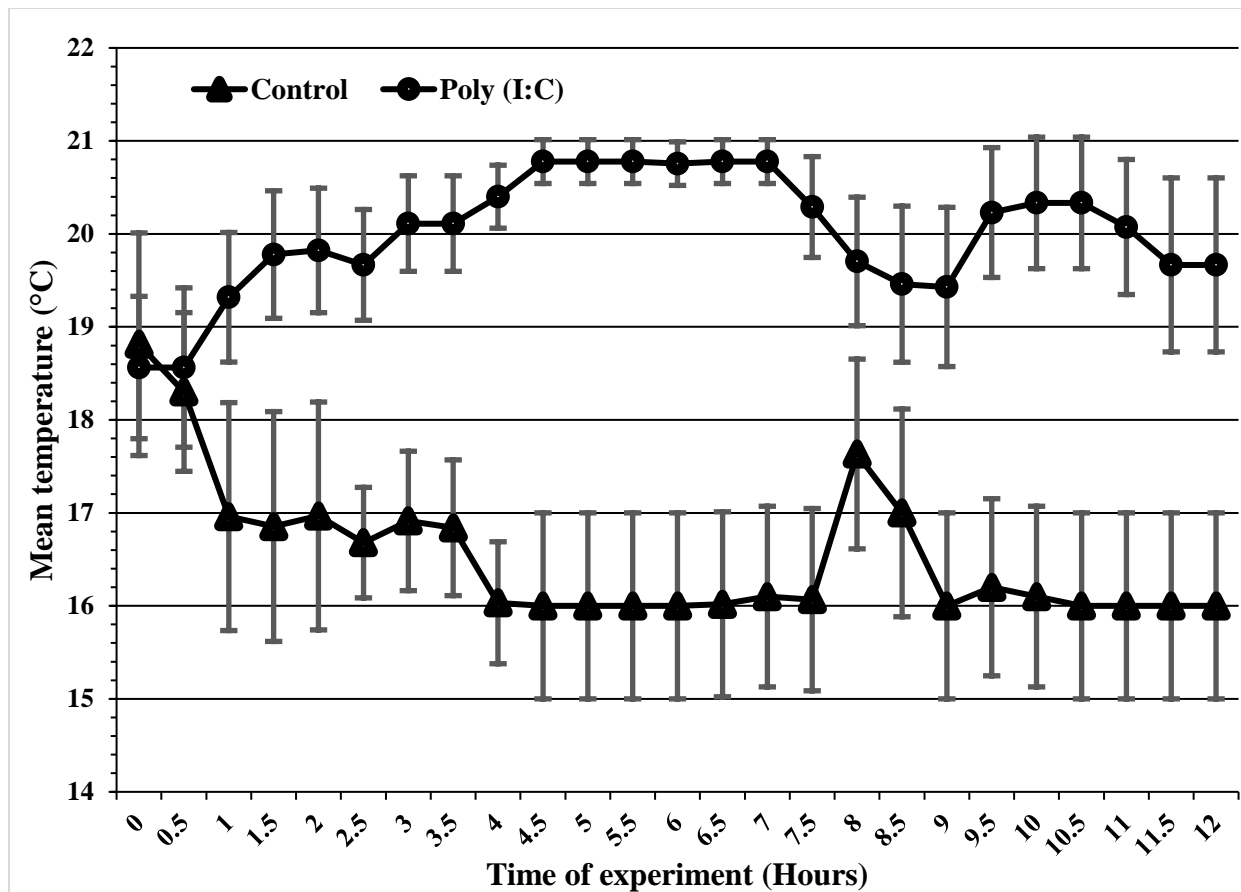

Figure 4: Average thermal preferences of *P. glutinosus* samples during the 12 h experiment in temperature chambers with standard error bars after treatment with Poly (I:C) (0.05 ml of dose 15 µg g-1 body mass).

The seasonal temperature preference comparison test between November and December showed no difference in temperature preferences between months (p = 0.728).

Discussion

The purpose of this study was to determine if *P. glutinosus* exhibits behavioral fever after exposure to the viral conditions of a synthetic pyrogen. We hypothesized that plethodontids would express behavioral fever based on past studies with other ectotherms and members of other select salamander families (Hutchison and Erskine, 1981, Sherman, 2008, and Parris et al. 2004). This hypothesis was supported as fever induced *P. glutinous* selected a higher-than-average temperature over control specimens in identical conditions. This finding supports previous evidence of salamanders utilizing ranges in environmental temperatures for regulation of behavioral fever and demonstrates that behavioral fever occurs specifically in the family Plethodontidae. These results can be used for the direct implication of future treatments of immunocompromised plethodontids in a biological or veterinary setting through use of temperature manipulation to reduce adverse effects or chance of mortality from pathogenic threats (Rakus et al. 2017).

These findings further support the necessity of expanding behavioral fever studies to investigate what factors alter how the process is used. This may prove easier than expected as the methodology presented provides a reliable animal model and pyrogenic model for consistent inductions of behavioral fever to eliminate confounding factors. Future long-term studies with plethodontids should investigate thermal gradients within population ranges and determine if thermal ranges are competitive or shared regions especially when a transmissible fever inducing component is considered (Da Silva Nunez and Jaeger, 1989 and Jaeger and Forester, 1993, Mathis, et al. 1990). Such studies could play a large role in understanding the behavior and ecology of plethodontids in a broader sense and contribute to establishing conservation zones for species in this family. Further investigations could involve direct investigation of how

plethodontid skin responds to direct contact with pathogens to see if this impacts behavioral fever response. This type of study could prove useful in determining the degree of immunity in the skin and may improve our understanding of how pathogens are transferred in populations between individuals and how long-term immunity from fatal pathogenic threats can be established.

Plethodontid reliance on environmental temperatures for a behavioral fever response when immunocompromised highlights the importance of considering how specialized amphibian groups such as plethodontids regulate their immune system. This consideration through future studies contributes to further understanding on how ectotherms deal with environmental stress and may prove vital towards establishing reliable conservation efforts. When considering the broad phylogenic history of behavioral fever there is a wealth of potential future contributions on the subject of behavioral fever in ectotherms. To summarize, the synthetic pyrogen Poly (I:C) was shown to effectively induce behavioral fever in *Plethodon glutinosus* through use of temperature chamber gradients. This supports evidence of Poly (I:C) as a fever inducer and supports the idea that the *Plethodontidae* family expresses environmental thermoregulatory behavior as a defense against immune threats.

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