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Termociclo diário ou regime térmico constante durante a exposição à alta temperatura: Efeitos sobre a ritmicidade comportamental, o estresse e a habilidade imunológica de rãs-touro americanas *Lithobates catesbeianus* (Shaw, 1802)

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Dedico a conquista desse trabalho às pessoas minorizadas que lutam para conseguir aquilo que é quase impossível aos olhos da sociedade. Ao conseguirem, representam muitas outras que também estão lutando.

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Ser biólogo é estar na fronteira do conhecimento.

E estar na fronteira do conhecimento significa ousar falar coisas.

(informação verbal)[1]

[1] Fala do Prof. Dr. Gilberto Fernando Xavier durante a aula introdutória da disciplina BIF0217, "Fisiologia Animal: Comunicação e Integração", para alunos de graduação em Ciências Biológicas do Instituto de Biociências da Universidade de São Paulo, 2018.

Resumo

A vulnerabilidade de anuros ao aquecimento global tem sido uma preocupação crescente. Comparando-se um cenário de temperatura constantemente elevada com o de variabilidade cíclica da temperatura ambiental na natureza, é razoável esperar que a variação diária da temperatura possa atenuar efeitos adversos do aquecimento ambiental sobre a aptidão, principalmente de ectotermos como os anfíbios. O presente estudo investigou os efeitos do estresse térmico sobre o comportamento e a fisiologia de machos juvenis de rãs-touro americanas (*Lithobates catesbeianus*) expostos a regimes de temperatura diária cíclica ou constante. As rãs de um ranário local foram trazidas ao laboratório (n = 42), mantidas sob ciclo claro-escuro natural, e foram alojadas individualmente em um sistema de recirculação de água com filtragem e controle termostático. Após aclimatação, foram expostas a regimes de temperatura constante a 24 °C (n = 21, T_{24}), constante a 30 °C (n = 9, T_{30}) ou cíclica diária de 24 a 30 °C (n = 9, $T_{24\text{-}30}$) por duas semanas. Ração para peixes e ninfas de baratas foram ofertadas três vezes por semana e a ingestão voluntária foi contabilizada para análise do comportamento alimentar. As rãs puderam alternar livremente entre submersão na água e exposição ao ar, e foram filmadas para análise da escolha de microhabitat e da atividade locomotora ao longo do dia. Níveis plasmáticos de corticosterona (CORT) e capacidade bactericida plasmática (BKA) foram medidos por ELISA e espectrofotometria, respectivamente. As rãs expostas a T_{24} permaneceram 60 % do tempo com o corpo na água e 38 % fora da água, indicando um uso diversificado dos microhabitats disponíveis. Por outro lado, os animais permaneceram 66 % fora da água quando expostos ao T_{30}, sugerindo que houve uma resposta de escape da menor disponibilidade de oxigênio e de outros prejuízos da água aquecida. O comportamento alimentar e a escolha de microhabitat não apresentaram padrão ritmico dia-noite consistentes em nenhum dos regimes. No entanto, o aumento de temperatura teve um efeito estimulador sobre a atividade locomotora, sendo essa mais concentrada durante o dia, em ambos os grupos e especialmente na fase de aquecimento do ciclo diário de temperatura em $T_{24\text{-}30}$. As rãs expostas a T_{30} e $T_{24\text{-}30}$ exibiram níveis mais altos de CORT e mais baixos de BKA em comparação a T_{24}, sugerindo que a temperatura de 30 °C causou estresse térmico associado a reorganização metabólica e disregulação da imunidade inata tanto no regime constante quanto cíclico. Assim, um prejuízo severo das funções fisiológicas e do desempenho dos animais poderia resultar da exposição prolongada a 30 °C em ambos os regimes, e mais estudos são necessários para elucidar os possíveis efeitos do aquecimento global no ambiente termicamente variável.

Palavras-chave: Aquecimento global. Termoperiodismo. Anfíbios. Rã. Fisiologia animal. Comportamento animal. Ritmos biológicos. Hormônios glicocorticóides. Imunocompetência.

Abstract

Anurans' vulnerability to global warming is a growing concern. When comparing a constantly increased temperature scenario to the cyclic thermal variability in nature, it is reasonable to expect that the daily variation in temperature could buffer adverse effects of environmental warming on fitness, especially of ectotherms such as amphibians. This study investigated the effects of thermal stress on the behavior and physiology of juvenile American bullfrog males (*Lithobates catesbeianus*) exposed to daily cyclic or constant temperature regimes. Frogs from a local farm were brought to the laboratory (n = 42), kept under a natural light-dark cycle, and individually housed in a recirculating water system with filtration and thermostatic control. After acclimation, the frogs were exposed to either constant 24 °C (n = 21, T_{24}), constant 30 °C (n = 9, T_{30}) or daily cyclic 24 to 30 °C (n = 9, $T_{24\text{-}30}$) temperature regimes for two weeks. Fish-fed and cockroach nymphs were offered three times a week, and voluntary ingestion was counted for feeding behavior analysis. The frogs could freely alternate between water submersion and air exposure and were filmed to analyze microhabitat choice and locomotor activity throughout the day. Corticosterone plasma levels (CORT) and bacteria-killing ability (BKA) were measured with ELISA and spectrophotometry, respectively. Bullfrogs under T_{24} stayed 60 % of the time submerged in water and 38 % of the time out of water, indicating a diversified use of available microhabitats. On the other hand, they spent 66 % of the time out of water under T_{30}, suggesting an escape response from the lower oxygen availability and other harms of warm water. Feeding behavior and microhabitat choice were poorly rhythmic in all regimens. However, there was an effect of higher temperature in enhancing locomotor activity during the day, suggesting an overriding effect of heat exposure for both groups, especially during the heating phase of the temperature cycle for $T_{24\text{-}30}$. Frogs under T_{30} and $T_{24\text{-}30}$ had higher CORT and lower BKA levels compared to frogs exposed to the T_{24} regimen, suggesting that 30 °C caused heat stress associated with metabolic reorganization and dysregulation of innate immunity in both constant and cyclic regimens. Thus, severe impairment of function and performance could possibly result of a more prolonged exposure of bullfrogs to 30 °C in either regimen, and further research is needed to elucidate the potential effects of global warming in the thermally variable environment.

Key-words: Global warming. Thermoperiodism. Amphibians. Bullfrog. Animal physiology. Animal behavior. Biological rhythms. Glucocorticoid hormones. Immunocompetence.

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List of Abbreviations

Abbreviation	Definition
ANOVA	Analysis of variance
BKA	Bacteria-killing ability
CEUA	Ethics Committee on the Use of Animals
CNS	Central Nervous System
CO_2	Carbon dioxide
CONCEA	National Council for the Control of Animal Experimentation
CORT	Corticosterone
ELISA	Enzyme-linked immunosorbent assay
GLM	Generalized Linear Model
GLMM	Generalized Linear Mixed Model
LMM	Linear Mixed Model
O_2	Oxygen gas (dioxygen)
pH	potential of hydrogen
T_{24}	Constant exposure to 24 °C water temperature
T_{24-30}	Cyclic exposure varying from 24 °C to 30 °C water temperature
T_{30}	Constant exposure to 30 °C water temperature

Summary

1

Introduction

In nature, temperature influences the behavior, the physiology, and the distribution of all organisms. The ectothermic animals are less effective at buffering body temperature against ambient temperature using physiological mechanisms, and their vulnerability to global climate change is a growing concern. For amphibians, the global raise in annual temperature may affect the animals at the individual, populational and community levels. However, it is unclear whether climate change directly affects amphibians' survival, by increasing body temperature and mortality rates, or indirectly affects other aspects of the organismal biology such as feeding rates and its interespecific interactions with competitors, predators, parasites, and disease to cause populational declines (BLAUSTEIN *et al.*, 2010; HUEY *et al.*, 2012).

The direct effects of temperature increase on ectotherms physiology span from biochemical reactions to behavior, but a full mechanistic understanding of the effects of temperature on biological processes across levels of organization is still lacking, as well as the adaptations that organisms use to cope with environmental heating created by human-induced climate change (SCHULTE, 2015). Because of a non-linear temperature effect on the rates of chemical reactions, some of the direct effects of high temperatures on energy expenditure are disproportionate and may disturb metabolic processes at subcellular, tissue, and organismal levels. For example, thermal performance curves describe the effects of rising temperature on protein configuration state and mitochondrial oxygen consumption, which affect the aerobic energy supply and organ function, leading to changes in whole-animal routine metabolic rate (HOCHACHKA; SOMERO, 2002; KERN; CRAMP; FRANKLIN, 2015; SCHULTE, 2015). Unlike most ectotherms, amphibians have very permeable and thin skin and their intrinsic high rates of evaporative water loss increases their susceptibility to dehydration outside of water when facing increased temperatures (GUEVARA-MOLINA; GOMES; CAMACHO, 2020; LILLYWHITE, 2006). Therefore, because amphibians depend on the aquatic environment, temperature effects on abiotic factors such as water evaporation, pH and gas solubility (*e.g.* O_2 and CO_2) can also directly affect protein stability, cutaneous water and gas exchange, and kidney function, ultimately affecting their abundance and distribution (BLAUSTEIN *et al.*, 2010; FREDA, 1986; HOCHACHKA; SOMERO, 2002; PIERCE, 1985; SHOEMAKER *et al.*, 1992).

The unpredictable temperature increase can also indirectly trigger thermal stress responses in ectotherms, and can even impair the immunological response to diseases if the stressor persists in the long-term (ROMERO; WINGFIELD, 2016). According to these

authors, there is a complex cascade of events involved in the perception, integration, and transduction of the environmental signals into chemical signals, performed by specialized cellular receptors, neural pathways, brain areas, regulatory neurosecretion in the Central Nervous System (CNS) and peripheral hormones, especially steroids, making the circulating levels of glucocorticoids as perhaps the most common index of stress (*e.g.* cortisol in fishes, and corticosterone in reptiles and amphibians). When the amphibians' CNS detect a stressor, such as temperature increase, the Hypothalamus-Pituitary-Interrenal axis releases corticosterone (CORT) into the plasma, and the hormone concentration returns to the baseline by negative feedback response. However, if the stressor persists and the animal becomes chronically stressed, CORT levels may become permanently elevated, resulting in a higher total amount of corticosteroids released and modulating functions of cardiovascular system, respiratory system, metabolism, and nervous system (NARAYAN *et al.*, 2019; ROMERO; WINGFIELD, 2016). It is also known that prolonged catecholamines release is integrated with other biological functions of the organism. For example, the exposure to long-term thermal stress reverberates in immunological parameters of ectotherms, such as immune-related gene expression, cytokine balance, proportion of cells that compose the innate and the adaptive immune system, phagocytic activity of blood leukocytes, and bacteria-killing ability of plasma, which may facilitate invasion by parasites and alter their susceptibility to disease (DHABHAR, 2014; DITTMAR *et al.*, 2014; HUO *et al.*, 2019; LIMA *et al.*, 2020, 2022). As a consequence, the decrease in immune capacity against parasites and diseases can alter the ecology of host-parasite systems, endorsing the importance of studies investigating the physiological impacts of temperature increase as a basis for conservation purposes of ectothermic species. Finally, at higher levels of biological organization, the environmental temperature reaches the behavioral pattern of ectothermic animals. In other words, besides the bottom-up kinetic effects of temperature increase, there are top-down integrated effects of thermal information perceived by the CNS, modulating behavioral changes such as microhabitat selection, basking pattern and daily activity pattern (ABRAM *et al.*, 2017).

Morash and colleagues (2018) argued that laboratory measures of performance and physiological variables conducted on animals acclimated to a thermally stable environment may not accurately reflect their performance in the wild, where temperature varies throughout day and night. According to these authors and Fabrício-Neto *et al.* (2019), ectothermic thermal physiology research often uses average conditions to understand the effects of temperature and simplify experimental designs, ignoring the fact that organismal responses to stable conditions may be quite different from the responses in variable conditions. According

to the Jensen's inequality effect, the difference between the animals' performance at an average temperature and across a range of temperature, for example, depends on the temperatures chosen and its relation to the animal's optimum temperature (MORASH *et al.*, 2018). The concerns about the effects of climate changes and global warming have led researchers to investigate ectotherms' ability to compensate changes in body temperature and survive overheating, using values of mean thermal tolerance, but ignoring that temperature may play a critical role in timing ectotherms' physiological processes, especially for those that are seasonal breeders and must take certain life history decisions to survive (BORAH *et al*., 2018; GUNDERSON; STILLMAN, 2015).

Interestingly, the studies that incorporated daily thermal fluctuations have shown that thermal variability can either favor or harm the development and performance of ectothermic animals, depending on the species, the average temperature, the amplitude of thermal variation and even the time of day when the thermocycle starts (ARRIGHI *et al.*, 2013; KERN; CRAMP; FRANKLIN, 2015; SPIELER *et al.*, 1977; VAJEDSAMIEI *et al.*, 2021). In fact, studies performed under daily cycling temperatures found out different responses across multiple levels of biological organization, including gene transcription, metabolism, stress response, immunity, larval development, growth, and reproductive maturation (ARRIGHI *et al.*, 2013; FABRÍCIO-NETO *et al.*, 2019; KERN; CRAMP; FRANKLIN, 2015; MORASH *et al*., 2018; PODRABSKY; SOMERO, 2004; SPIELER *et al.*, 1977; TERRELL *et al.*, 2013; VAJEDSAMIEI *et al.*, 2021; VILLAMIZAR *et al*., 2012). According to Morash *et al.*, (2018), the use of more realistic temperature profiles in experimental biology must be encouraged to improve our understanding of the temperature effects and to enable more realistic predictions about the effects of climate change on ectotherms. Additionally, as climate changes decrease the predictability of environmental conditions correlated with the photoperiodic cycle (such as food availability), and temperature itself can act as a non-photic synchronizer for ectotherms, understanding the animals sensitivity and ability to respond to non-photoperiodic environmental cues can favor ecological and conservation approaches based on their survival, especially for younger developmental stages of the life cycle (CANALE; HENRY, 2010; HUEY; KINGSOLVER, 2019; LÓPEZ-OLMEDA, 2017).

The amphibians have been intensively used in studies about the impact of human activities on ecological systems, such as global climate changes. The estimation of the magnitude of the impact of climate on metabolic rate of amphibians have shown that tropical species live closer to their upper thermal tolerance limit and should experience a larger metabolic shift than species from temperate or Artic zones, even though the temperature

change in the tropics has been relatively small (DILLON; WANG; HUEY, 2010; GUNDERSON; STILLMAN, 2015; SOMERO, 2010). From an ecological point of view, tropical endemic amphibians become more threatened than cosmopolitan or invasive species, stimulating focal conservational actions (CORDIER *et al*., 2019; GARCÍA; ORTEGA-HUERTA; MARTÍNEZ-MEYER, 2014). Yet, the use of local invasive species as the cane toad *Bufo marinus* or the bullfrog *Lithobates catesbeianus* is suitable for physiological studies and a viable alternative for laboratories with limited resources, considering the advantageous availability of species easily bred in laboratory settings (BURGGREN; WARBURTON, 2007; HOPKINS, 2007). The present study aimed to investigate the effects of high temperature exposure in juvenile *Lithobates catesbeianus* by using a recirculating water system. We focused on chronobiological, behavioral, hormonal and immunological responses to increased temperature under continuous exposure and with daily cyclic variation. More specifically, we measured the feeding behavior, the locomotor activity behavior, the microhabitat choice, and the plasma corticosterone levels and bacteria-killing ability in groups of bullfrogs exposed to daily thermal cycles and exposed to constant temperatures. We hypothesized that the fluctuating temperature in the cyclical regimen would attenuate the effects of hyperthermia on corticosterone levels and bacteria-killing ability of plasma, compared to the effects in the constant regimen. Additionally, the exposure to high temperature would intensify frogs' activity and duration of air exposure only at the light and warmer phase of the day in the cyclic regimen, and regardless of the phase of the day in the constant regimen.

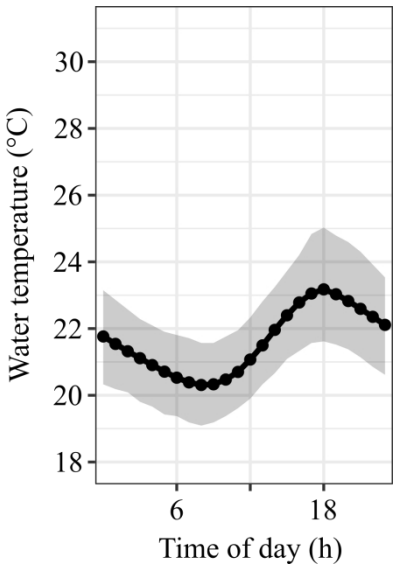
Materials and Methods

2.1 Experimental animals

In the present work, we used 42 juvenile male individuals of *Lithobates catesbeianus* (Shaw, 1802) purchased from a local farm in São Paulo - SP, Brazil (*Rã's World* - 23°28′4.8″S, 46°42′7.2″W). Frogs with a body mass ranging from 130 to 288 g were brought to the laboratory at the University of São Paulo during summer in December 2019 (n = 24) and January 2020 (n = 18). The frogs were immediately transferred to a large box of 180 liters containing stones and dechlorinated water up to the vocal sac, where they were kept for 24 to 48 hours at room temperature around 21 °C. This procedure allowed the elimination of waste products from digestion before transferring frogs to the buckets in the experimental system (Section 2.2).

The daily variation of water temperature in the frogs' farm was recorded with a HOBO data logger (Onset, Pendant temp) during 27 days in the summer of 2020. As shown in Figure 1, the temperature exhibited a gentle cycle with daily mean of 21.62 °C ± 1.88 °C, and the lowest and the highest values registered of 18.33 °C and 26.49 °C, respectively. This information was used to validate the temperatures in the experimental regimens (Section 2.3.1).

Figure 1 – Daily variation of water temperature in the frog´s farm

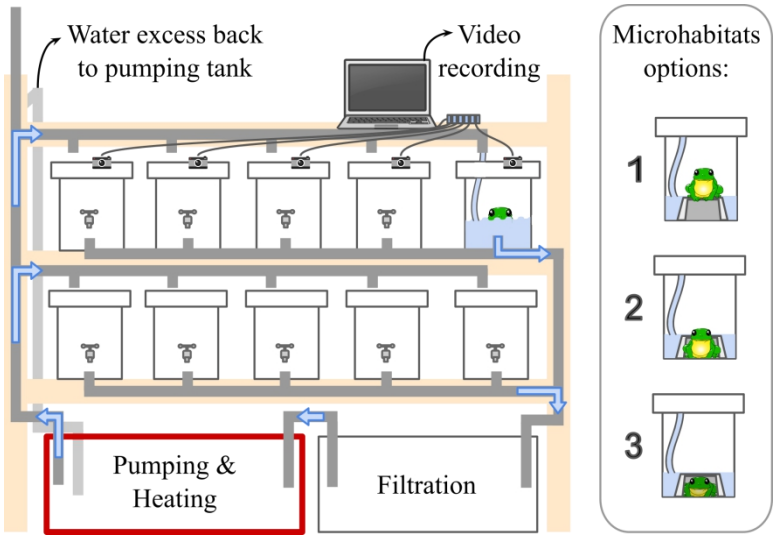


Water temperature varied around a mean of 21.62 ± 1.88 °C between 23 February and 20 March 2020.The mean minimum was 20.30 °C at 8 h, and the mean maximum was 23.17 °C at 18 h. The black dots indicate the mean value and the gray area shows the interquartile range for each hour of the day.

2.2 Recirculating water system and temperature control

The experiments were performed using a recirculating water system built on two wooden storage racks, each one with two shelves (Figure 2). Frogs were individually housed inside buckets, a total of 10 frogs in Rack 1 (190 cm height × 44 cm depth × 180 cm width) and of 14 frogs in Rack 2 (190 cm height × 44 cm depth × 245 cm width). The water at a proper temperature was pumped up from a reservoir on the floor and reached the top, where a spillway dissipated the excess flux. The water moved horizontally and downwards in a pipe system and flowed into 20-liter buckets aligned in parallel sequence on the shelves. The incoming water flow rate was adjusted on each bucket entry to roughly 600 ml/min, and the water flowed out of buckets continuously. A volume of water equivalent to an eight cm high column was retained inside the buckets, allowing the frogs to submerge partially or entirely. The water flowed from the buckets into a 130 to 180-liter plastic box containing a filtering system on the floor. The filtering system was composed of acrylic felt (mechanical filtering), K1 bio-media in motion (biological filtering), activated charcoal (chemical filtering), and an ultraviolet lamp (Ace Pet, 24W). The filtrated water flowed through a U-shape siphon into the pumping reservoir, where the cycle restarted. The same reservoir provided water temperature control, as detailed below.

Figure 2 – Diagram of the recirculating water system with temperature control used in experiments with *L. catesbeianus* frogs



On the left side, the diagram illustrates one wooden rack used to support a PVC pipe system (dark gray) and to house ten bullfrogs individually in a series of buckets. The water was pumped upwards from a reservoir on the floor (highlighted in red) and circulated through pipes and buckets according to the path indicated by blue arrows. Outgoing water from buckets flowed into the filtration reservoir on the floor and then to the pumping reservoir, where the cycle restarted. On the right side, the diagram illustrates the microhabitats available inside the buckets that were used to study frogs' behavior by video recording (See Section 2.4.2).

Small plastic boxes (7 cm height × 11 cm inferior diameter × 13.5 cm superior diameter) were glued upside down on the buckets' bottom, and an opening was cut in the lateral wall allowing the frogs to get inside and use for shelter. The frogs could freely alternate among three microhabitats inside the buckets: total emersion and air exposure on the top of the box, partial submersion in water around the box, or total submersion in water inside the box (Figure 2).

Aquarium heaters (Atman, Hopar, Minijang, and Hagen) performed the temperature control. During exposure to constant temperatures, pipes and reservoirs were covered with plastic bubbles to minimize heat loss, and the heaters were always on. During cyclic temperature exposure, the heaters plugged into a timer alternated between on and off phases within a day, with the heating phase synchronized with the light phase. Additionally, heat loss during the night was promoted by an air conditioner constantly set to 21 °C, which led to slower temperature changes during the cooling phase. Digital thermometers (DS18B20), plugged into an Arduino Uno board, were placed at multiple locations in the system to continuously register water and air temperature, as detailed in the next section. Water and air temperatures were also measured occasionally with mercury thermometers (Incoterm), and cross-checked with temperatures measured with digital thermometers to correct any large deviations caused by sensor inaccuracy or electronic noise.

2.3 Experimental design and treatment

2.3.1 Temperature regimes

Initially, one group of frogs (n = 24) was housed in Racks 1 and 2 for one week acclimation to laboratory conditions, during which the target water temperature was set at 23 °C. Following the acclimation period, frogs stayed under these conditions for one more week for data collection (n = 21) (Figure 3). This group of frogs died from overheating due to an automatic temperature control failure, and another group was used in the next steps of the protocol.

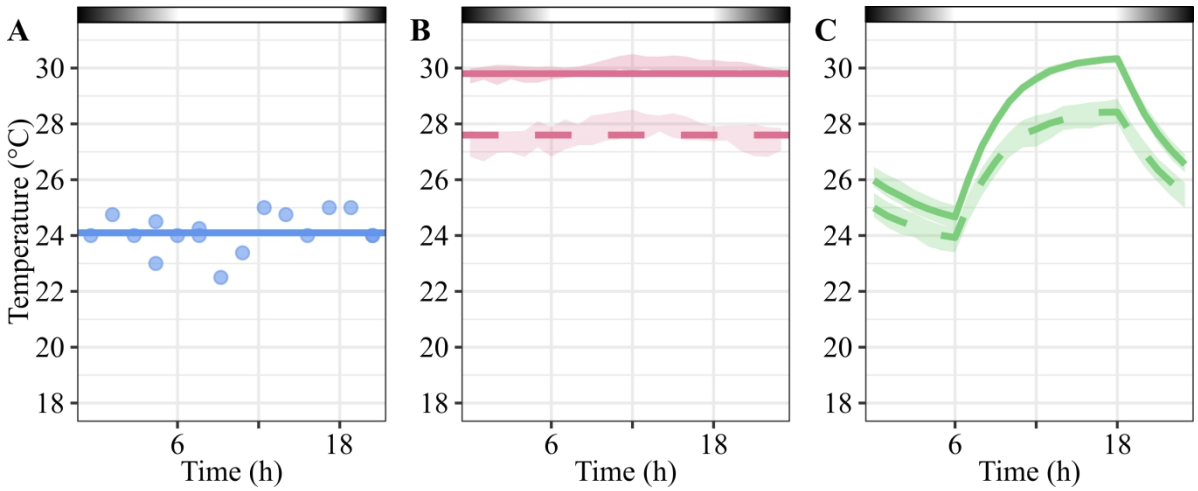
The second group of frogs (n = 18) was housed and acclimated to the experimental conditions as above (one week at 23 °C). Frogs in Rack 1 (n = 9) were exposed to 30 °C in a constant regimen, and frogs in Rack 2 (n = 9) were exposed to 30 °C in a cyclic regimen, for two weeks. The target temperature of 23 °C could not be reached either in constant or cyclic regimen with the equipment available. The equipment settings and recorded temperature of each thermal regimen are summarized in Table 1.

Table 1 – Summary of equipment settings and temperature regimens used in experiments with *L. catesbeianus* frogs. The heaters were set according to target temperatures, and the water temperature of each experimental regimen was continuously recorded for two weeks.

Group	Temperature regimen	Target temperature	Heating power	Operation mode	Recorded temperature (median ± SD)
1	T_{24}	23 °C	150 W (Rack 1) 200 W (Rack 2)	Always on	24.0 ± 0.7 °C
2	Acclimation of T_{30} and $T_{24\text{-}30}$	23 °C	150 W (Rack 1) 200 W (Rack 2)	Always on	23.4 ± 0.3 °C
	T_{30}	30 °C	350 W (Rack 1)	Always on	29.9 ± 0.4 °C
	$T_{24\text{-}30}$	Cyclic 23-30 °C	500 W (Rack 2)	On from 6 h to 18 h	24.4 ± 0.7 °C at 6 h 30.2 ± 0.3 °C at 18 h

Figure 3 shows water and air temperatures inside buckets that were recorded during the experimental period, with target temperatures set at 23 °C constant (T_{24}), 30 °C constant (T_{30}), and 23-30 °C cyclic ($T_{24\text{-}30}$). The mean air temperature near the flat surface of the box inside the bucket was slightly lower than the water temperature in all thermal regimens. Measurements performed with agar models suggested that the body temperature of frogs entirely submerged in water tended to be similar to the water temperature and at an intermediate value between water and air temperatures when frogs are out of water (Lidia S. YANO, unpublished results; See the Appendix A for details).

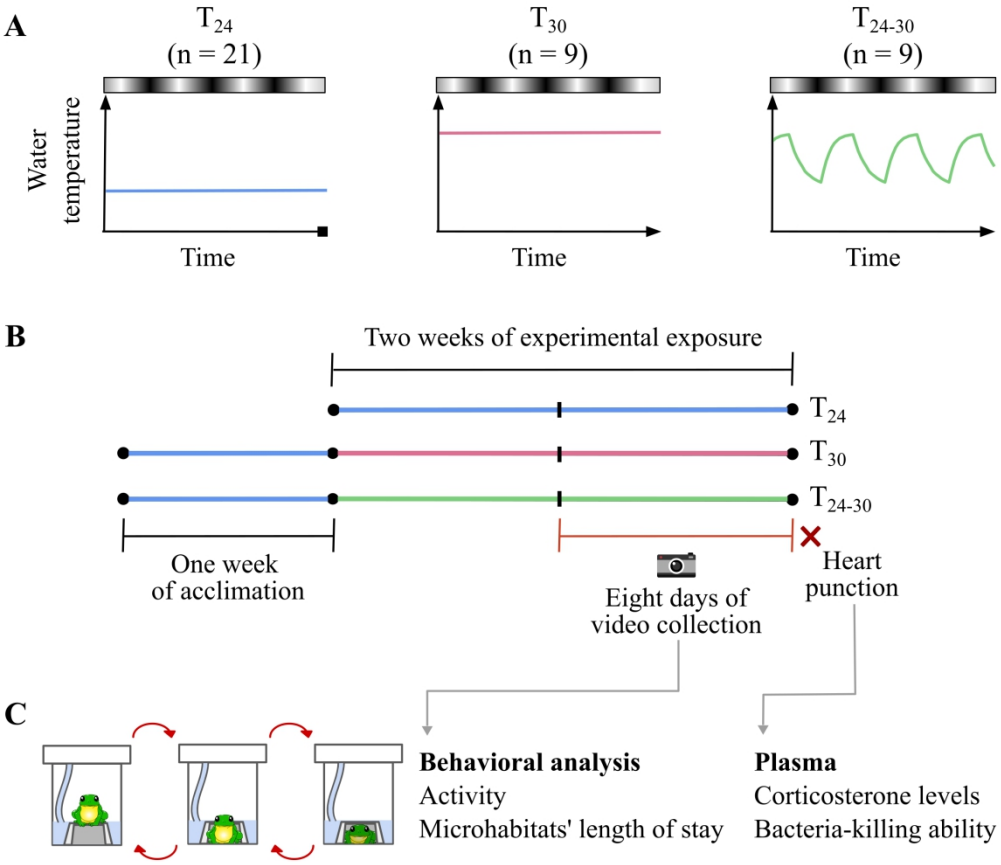
Figure 3 – Daily water and air temperature variation inside buckets during exposure of *L. catesbeianus* frogs to constant (T_{24}, T_{30}) and cyclic ($T_{24\text{-}30}$) temperature regimens



Blue values indicate the daily water temperature variation in the T_{24} regimen, with punctual measures with mercury thermometers (blue points) and the median water temperature (blue line). Pink areas correspond to the T_{30} regimen and green areas to the $T_{24\text{-}30}$ regimen, with the daily variation of water (solid) and air (dashed) temperatures and interquartile ranges (smooth areas). The black and white bar above the graph represents the light (white), dark (black), and dawn/dusk (gray) phases of the natural photoperiod. The night phase of the light-dark cycle coincides with the cooling phase of the $T_{24\text{-}30}$ regimen.

According to the protocol, groups of frogs were exposed to T_{24}, T_{30}, or $T_{24\text{-}30}$ regimens during two weeks, however, the total stay in the laboratory varied among the groups. The first group (T_{24}) stayed for two weeks, and the second (T_{30} and $T_{24\text{-}30}$) stayed for three weeks due to the acclimation period (Figure 4). Data collection was intended to allow the analysis of daily patterns of feeding behavior, locomotor activity, and microhabitat choice under each experimental regimen (Sections 2.4.1 and 2.4.2), and by the end of exposure, blood samples were taken by cardiac puncture for later analysis of plasma corticosterone and bacteria-killing ability (Sections 2.4.3 to 2.4.5). In the end of the experimental period, frogs of the T_{24} group were killed by overheating due to failure of the automatic temperature control. Frogs of the T_{30} and $T_{24\text{-}30}$ groups were decapitated, and tissue samples were stored frozen for later analysis in a collaborative study (Lidia S. YANO, unpublished results).

Figure 4 – Experimental design in the study of *L. catesbeianus* frogs exposed to constant (T_{24}, T_{30}) and cyclic ($T_{24\text{-}30}$) temperature regimens



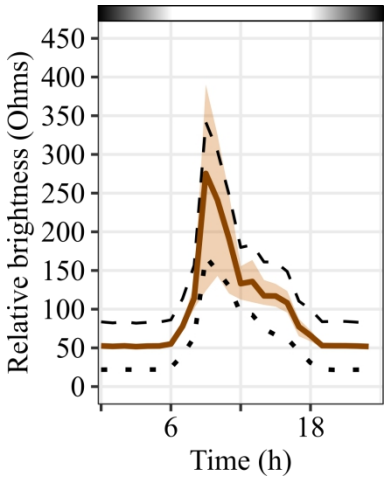
A – Temperature regimens and experimental groups: T_{24} (n = 21), T_{30} (n = 9), and $T_{24\text{-}30}$ (n = 9). The black and white bars above each graph represent the light (white), dark (black), and dawn/dusk (gray) phases of the natural photoperiod. B – Timeline of acclimation, exposure to temperature regimens, and data collection. C – Behavioral and physiological data collection (Section 2.4). The number of transitions between microhabitats and the length of stay at each microhabitat were used in the analysis of locomotor activity and microhabitat choice. The plasma samples were used in corticosterone and bacteria-killing ability assays.

2.3.2 Natural light and dark cycle

The experiments were performed indoors during summer, with windows providing natural light and dark cycles. Two photoresists (5 mm LDR), also plugged into the Arduino Uno board, monitored the relative brightness of the room continuously. The natural light in the room exhibited a peak around 9 h, and the mean brightness between 8 h and 16 h was at least double the value found during the night phase (Figure 5).

Two red lamps (A60, 5 W red light, Lexman, and TKS 14-1, 14 W, Taschibra) were switched on uninterruptedly in the laboratory during the experiments to facilitate night vision during feeding and frogs inspection, aiming to minimal interference for the animals (Section 2.3.3). Additionally, the experimenters used headlamps covered with red acetate when entering the room at night, using cellphone screens with a blue-light filter application (Twilight, set to 1000 K, 100 % intensity, and 65 % opacity). All equipment in the room that emitted light was covered with red acetate sheets (*e.g.*, laptop screens; Section 2.4.2).

Figure 5 – Daily variation of brightness in the room during the experiments



The black and white bar above the graph represents the light (white), dark (black), and dawn/dusk (gray) phases of the natural photoperiod. Dotted and dashed lines show the mean photoresistance in the room measured by vertical and horizontal sensors, respectively. The orange values indicate the average (solid line) and the interquartile range (shadow) of daily brightness variation measured by the two sensors.

2.3.3 Animal feeding and checking routines

The frogs were individually fed once a day and twice a week with pellets of fish commercial food (*Poli-Peixe 400 Intensivo*), using an amount that corresponds roughly to 1 % of the body mass per feeding event. The pellets were put into the buckets through a hole on the lid (5 cm diameter) with minimal disturbance. After 15 minutes, the leftover food was carefully removed with an aquarium net. Additionally, nymphs of the cockroach *Blaptica dubia* were offered on another day of the week, respecting the interval of two or three days between meals. The nymphs weighing around 1 g were manually selected from a laboratory colony and used to encourage normal feeding behavior and as nutritional support (HADFIELD; CLAYTON; BARNETT, 2006). The frogs were individually weighed weekly to track changes in body mass during the experiments.

The animals and the components of the recirculating water system were inspected daily to check for signs of disease or equipment malfunction. Feeding and checking routines were distributed across the day to reduce the effects of food ingestion or human presence on the frogs' daily activity pattern. Feeding events were distributed around four equal intervals within the 24 hours interval while checking events were distributed around three intervals from 8 h to 18 h for better vision. During the experiments, the water flow rate gradually decreased in the system, from 484 ± 78 ml to 360 ± 117 ml per minute over approximately two weeks, probably due to accumulation of organic matter in the water pipe and valves inefficiency. Both pH and ammonia levels remained stable during the experimental period as presented in Table 2.

Table 2 – Descriptive values of the water pH, ammonia levels, and flow at the recirculating water system during the experiments, taken as parameters of water quality, measured between 22 January 2020 and 21 February 2020.

Parameter	N	Average	SD
pH	12	5.23	± 0.23
Ammonia (mg/l)	12	6.64×10^{-6}	$\pm 1.55 \times 10^{-5}$
Water flow (ml/min)	72	442	± 130

2.4 Data collection

2.4.1 Feeding behavior

The total amount of food ingested per individual frog was monitored at each feeding event during the exposure to T_{24} (n = 21), T_{30} (n = 9), and $T_{24\text{-}30}$ (n = 9) regimens, and used to estimate the rate of feeding success and the voluntary food intake. The rate of feeding success was calculated by the ratio between the number of times that food was ingested and the total number of trial and values were expressed as percentage. The relation between the rate of feeding success and the time of the day that food was offered was used to analyze the temporal organization of feeding behavior. The voluntary food intake was calculated by subtracting the amount of food residues in the buckets from the amount of food offered in each event, and values were expressed in grams.

2.4.2 Video recording and analysis of locomotor activity and microhabitat choice

The behavior of frogs during exposure to T_{24} (n = 12), T_{30} (n = 5), and $T_{24\text{-}30}$ (n = 7) regimens was video recorded for eight days (Figure 4B). In each rack, small clip webcams (XHC Camera) were attached to the lid of the buckets (Section 2.3.3) and plugged into a USB Hub in a laptop (Linux Ubuntu 18.04 LTS). The webcams were configured with a "bash" script so that one photograph was taken per second, alternating one bucket at a time. Black plastic symbols were glued to each bucket to allow frogs' identification. Another "bash" script ordered the image files and assembled videos of each day recorded of each animal.

The images were analyzed using VLC Media Player (3.0.9.2 version), and frogs behavior was described by the locomotor activity and microhabitat choice inside buckets (Figure 4C). The count number of transitions between microhabitats was taken as a proxy of locomotor activity, and the mean number of transitions during eight days, per individual frog, was used to describe the daily amplitude of the locomotor activity. The length of time spent by each frog in total emersion, partial submersion, or total submersion inside the box was taken as a proxy of microhabitat choice. The length of time in each microhabitat was calculated as the mean percentage time relative to the total recording time during eight days. All video excerpts that coincided with human presence in the laboratory, as explained in Section 2.3.3, were disregarded in the behavior analysis.

2.4.3 Blood samples collection and processing

At the end of the exposure to the temperature regimens (Figure 4B), 300 µl to 400 µl of blood was taken from each frog by heart puncture using 1 ml heparinized (10 % solution) syringes with 13×0.45 mm needles. The blood was centrifuged at 600 *g*, 10 °C, for 4 minutes to obtain the plasma, which was stored at −80 °C for bacteria-killing ability (BKA) and corticosterone (CORT) assays (Sections 2.4.4 and 2.4.5). The puncture was always performed from 13 h to 15 h to ensure that both T_{30} and $T_{24\text{-}30}$ groups of frogs were at the closest instantaneous temperature, and the handling time duration was annotated.

2.4.4 Analysis of plasma corticosterone levels (CORT)

The plasma samples were used 5 to 596 days after the plasma collection to extract steroid hormones with 3 ml of ether. Then, the CORT levels were measured by enzyme-linked immunosorbent assay (ELISA, Cayman Chemical), according to the manufacturer's instructions, and the samples' absorbance was red at λ = 405 nm (Spectra Max 250).

2.4.5 Analysis of plasma bacterial-killing ability (BKA)

The BKA assays were performed 21 to 22 days after the plasma collection, following the protocol of de Assis *et al.* (2013). Briefly, plasma solutions (10 µl diluted in 190 µl Ringer solution for amphibians) were incubated with 10 µ solution of *Aeromonas hydrophila* grown overnight (2.5×10^7 ml^{-1}) at assay temperatures of 24 °C, 27 °C, and 30 °C. Plasma samples interacted with the bacteria until the bacteria control without plasma reached around 50 % of optical density (Spectra Max 250, λ = 595 nm). BKA values represent the proportion of bacteria cells killed by the samples at the three assay temperatures and were calculated according to the formula: *1 - (sample optical density ÷ bacteria control optical density).*

2.5 Statistical analysis

Data exploration and statistical analysis of locomotor activity, microhabitat choice, feeding success, voluntary food intake, BKA, and CORT levels were performed in R (4.1.2 version). The data were explored according to the protocol proposed by Zuur, Ieno and Elphick (2010), then submitted to a Shapiro-Wilk normality test (stats package), to linear models approach (lme4 package), and finally, to *post hoc* Tukey's test (emmeans package).

The temperature regimen was an explanatory variable for all linear models. Daily values of the locomotor activity, the time spent in each microhabitat, the rate of feeding success, and the voluntary food intake were analyzed as a Generalized Linear Model (GLM).

The first and the last values of frogs' body mass were compared with a repeated measures Student's *t*-test, and only the rate of feeding success and the voluntary food intake included the animal's body mass to the GLM explanatory variables. The light and dark phases or the temperature phase in the cyclic regimen (0-6 h, 6-12 h, 12-18 h, and 18-24 h) were included in the analysis of locomotor activity, length of stay at each microhabitat, and rate of feeding success, and the patterns of temporal organization of microhabitat choice and feeding success were analyzed using Linear Mixed Models (LMM) to control the random same-individual effect. They tested the interaction between phase and temperature regimen in the explanatory variables. The daily pattern of locomotor activity was similarly analyzed using Generalized Linear Mixed Models (GLMM).

The BKA and CORT levels were analyzed using the LMM approach. The assay temperature was added in BKA analysis to test the interaction between temperature regimen and assay temperature, besides controlling the random same-individual effect. The duration of handling and the temperature regimen were added in CORT analysis as explanatory variables. The animal spatial position in the room was included in the analysis of random effects, to control potential stressors such as the noise of the air conditioner or the pumping and heating equipment in the recirculating water system.

The model selections were performed through ANOVA nested linear models comparison, using $p < 0.05$ as the threshold to retain explanatory variables. When p values were near 0.1, Tukey's test was performed to assure that statistical differences between groups would not be ignored if the model was reduced. The *p*-value in the GLM models comparison referred to Fisher statistics. In contrast, the *p*-value in LMM and GLMM models comparison referred to Chi-squared statistics using Maximum Likelihood estimations. After the minimal adequate models were defined, their residual plots were verified. Both formulas and residual plots of the models used are available in the Appendix C.

2.6 Ethical approval

This study was approved by the Ethics Committee on the Use of Animals (CEUA) from the Biosciences Institute of the University of São Paulo (protocol ID 340/2018). The Committee's approval referred to the frogs' purchasing, housing, caring routine, experimental treatments, data collection, and decapitation, following the Brazilian Guide to Good Practices for Euthanasia in Animals (2013) and the Euthanasia Practice Guideline of the National Council for the Control of Animal Experimentation (CONCEA)(2018).

Results

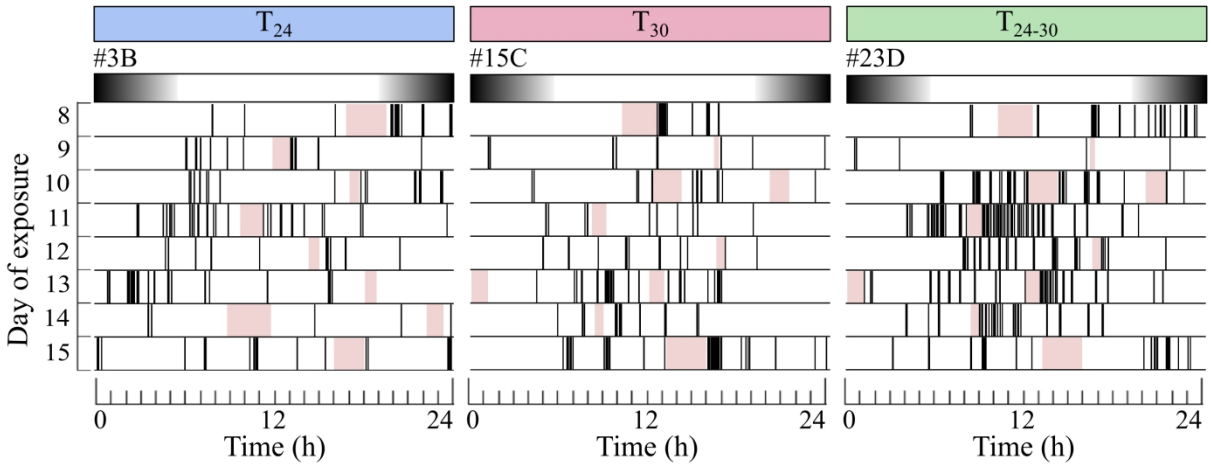
3.1 Experimental system and animal welfare

The target temperatures were reached and kept stable during the experimental period in the T_{24} and T_{30} regimens. In the $T_{24\text{-}30}$ regimen, maximum temperatures around 30 °C were quickly reached in 6 hours and stabilized at this value for the next 6 hours; otherwise, the minimum temperature around 24 °C was slightly higher than expected and it was reached in 12 hours due to the slow rate of passive cooling in the system. In contrast to the laboratory conditions, the water in the farm where the bullfrogs were cultivated exhibited much smaller rates of heating and cooling during summer, around 2 °C in 12 hours, besides reaching minimum and maximum daily temperatures lower than in the experimental regimens. During the study, the bullfrogs had no signs of disease or natural mortality, an indicator of good quality of water in the recirculating system. Besides, their body weight was unchanged during exposure to the experimental regimes ($p = 0.7339$, $p = 0.4581$ and $p = 0.3906$ for T_{24}, T_{30} and $T_{24\text{-}30}$ regimens, respectively), and they showed the typical behavior of live prey capture that is considered an indication of healthy nutritional state (HADFIELD; CLAYTON; BARNETT, 2006).

3.2 Locomotor activity

Representative actograms of individual frogs are shown in Figure 6, where each transition between microhabitats was plotted along the days of observation to illustrate the daily locomotor activity pattern during exposure to each thermal regimen. Actograms of all individuals are available in the Appendix B.

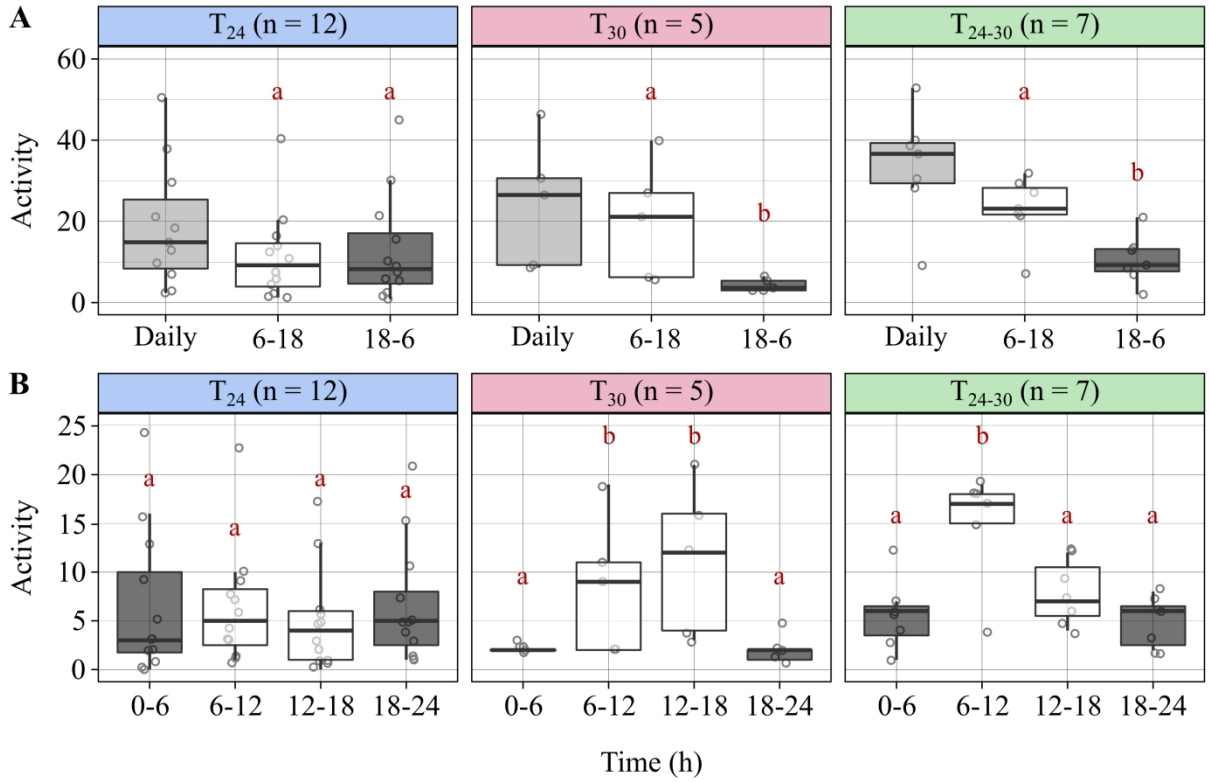
Figure 6 – Representative actograms of daily locomotor activity patterns of *L. catesbeianus* frogs chronically exposed to constant (T_{24}, T_{30}) or cyclic ($T_{24\text{-}30}$) thermal regimens



The bars on the top of the actograms indicate the natural photoperiod variation, and the night phase of the light-dark cycle coincides with the cooling phase of the $T_{24\text{-}30}$ regimen. Vertical marks indicate transitions between microhabitats. Pink areas indicate the human presence during feeding and checking routines, and were disregarded in the analysis.

There were no differences in the mean daily locomotor activity of frogs among the constant (T_{24}, T_{30}) and cyclic (T_{24-30}) temperature regimens ($p = 0.6179$). On the other hand, the amount of activity of frogs exposed to T_{30} and T_{24-30} regimens was specifically higher in the interval of 6 h to 18 h (light) than in the interval of 18 h to 6 h (dark), indicating an effect of the phase of the day ($p < 0.0001$; Figure 7A). This rhythmic behavior was not observed in the T_{24} regimen ($p = 0.8981$), suggesting that frogs exposed to temperature increase expressed a day-night activity pattern. Interestingly, the amount of activity was higher only during the heating phase for frogs in the T_{24-30} regimen, more specifically from 6 h to 12 h, compared to 12 h to 18 h ($p = 0.0041$) and to the other time intervals ($p < 0.0001$; Figure 7B). This pattern suggests that acute response to higher temperature might be involved in the microhabitat transition of frogs exposed to the thermocycle.

Figure 7 – Locomotor activity of *L. catesbeianus* frogs chronically exposed to constant (T_{24}, T_{30}) and cyclic (T_{24-30}) thermal regimens

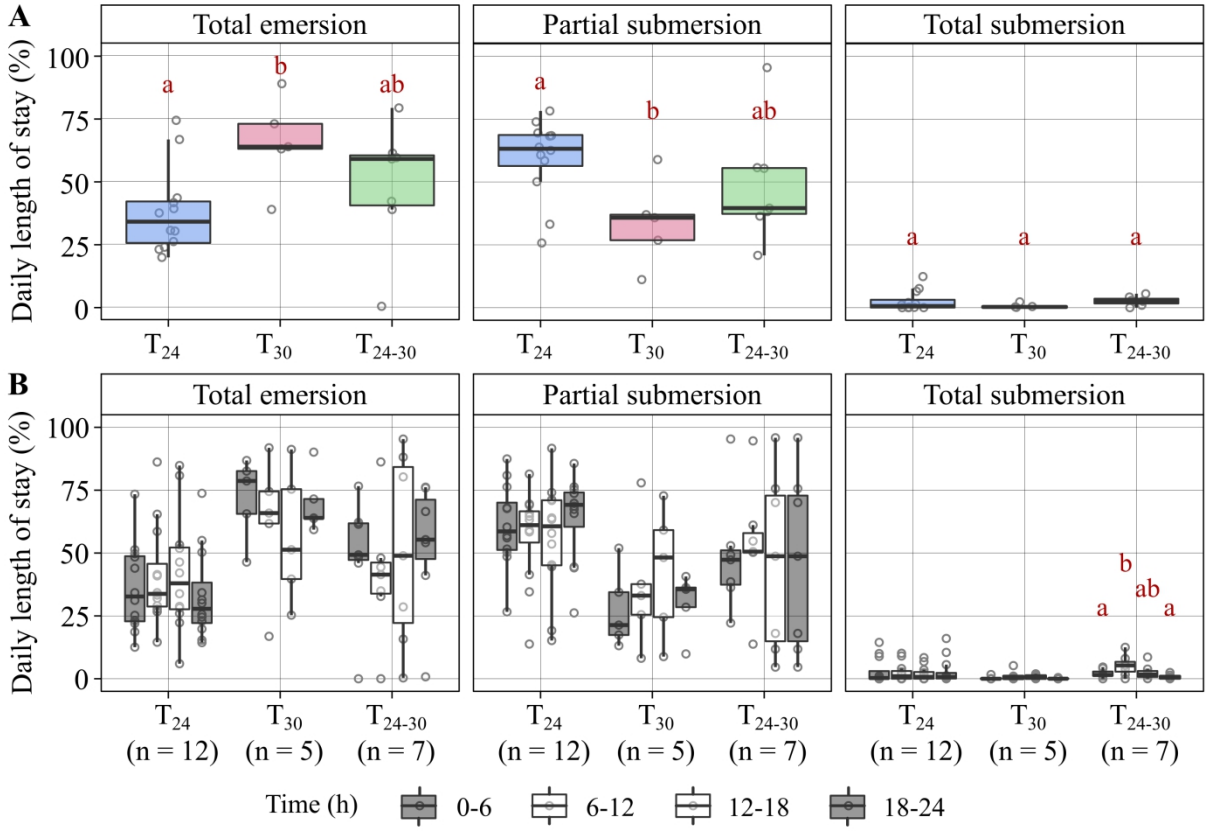


Empty circles represent the individual data, corresponding to the mean of all locomotor activity events detected in each time interval throughout the eight days of data collection. A – Daily activity (light gray boxes) and activity partitioned from 6 h to 18 h (white boxes) and from 18 h to 6 h (dark gray boxes). B – Locomotor activity is partitioned into four time intervals of the day, of which 6 h to 12 h corresponds to the heating phase in the T_{24-30} regimen. Different letters indicate statistical differences for Tukey's pairwise comparisons between phases, at $p < 0.05$.

3.3 Length of stay and microhabitat choice

The effect of different thermal regimens on the length of stay at the available microhabitats is presented in Figure 8. The daily length of stay at each microhabitat did not differ between frogs exposed to T_{24} and $T_{24\text{-}30}$ regimens. However, frogs exposed to the T_{30} regimen spent much more time out of water than when to the T_{24} regimen ($p = 0.0332$). In a complementary way, their length of stay partially submerged was significantly shorter compared to the T_{24} regimen ($p = 0.0359$; Figure 8A). Interestingly, the T_{30} frogs preferred total emersion instead of partial submersion compared to T_{24} frogs regardless of the phases of the day ($p = 0.0495$ and $p = 0.0540$, respectively). In general, the frogs spent only a minor fraction of the daytime in total submersion, where the daily length of stay was similar among the thermal regimens ($p = 0.3242$). However, frogs exposed to $T_{24\text{-}30}$ stayed longer in this condition during the day, specifically during water heating from 6 h to 12 h compared to 18 h to 24 h ($p = 0.0009$) and 0 h to 6 h ($p = 0.0506$; Figure 8B). Graphics of the daily microhabitat choice of all animals are available in the Appendix B.

Figure 8 – Length of stay and microhabitat choice in *L. catesbeianus* frogs during exposure to constant (T_{24}, T_{30}) and cyclic ($T_{24\text{-}30}$) thermal regimens

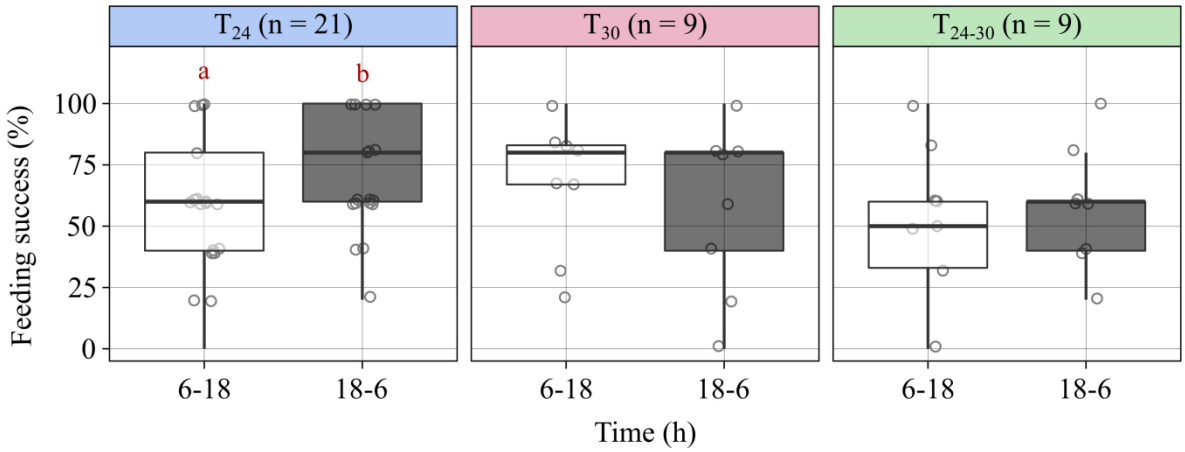


Values are percentage of time relative to daily observation, and empty circles represent individual mean data calculated from 8-day measurements. A – Daily length of stay at each microhabitat under T_{24} (blue), T_{30} (pink), and $T_{24\text{-}30}$ (green). B – Daily length of stay at each microhabitat was partitioned into four time intervals of the day, of which 6-12 h corresponds to the heating phase in the $T_{24\text{-}30}$ regimen. Different letters indicate statistical differences for Tukey's pairwise comparisons between groups or phases, at $p < 0.05$.

3.4 Feeding behavior

Bullfrogs generally showed a high acceptance of the food offered during the experiments. The animals voluntarily ingested an average of 88 % of cockroaches, 46 % of fish fed, and 56 % of the total offered, and no differences were found among the thermal regimens (p = 0.1306). The feeding success depended on the phase of the day only under the T_{24} regimen (Figure 9). The values were higher in the dark than in the light phase under the T_{24} regimen (p = 0.0512), especially from 18 h to 24 h. In contrast, there was no clear dependency on the phase of day under T_{30} (p = 0.8722) and $T_{24\text{-}30}$ (p = 0.7924) regimens, suggesting an effect of high temperatures on weakening the daily pattern of feeding behavior.

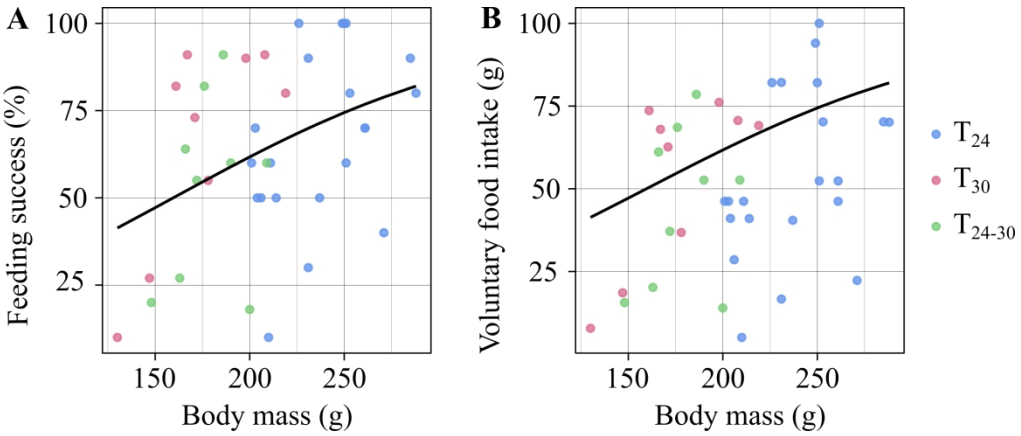
Figure 9 – Daily pattern of feeding success of *L. catesbeianus* frogs chronically exposed to constant (T_{24}, T_{30}) and cyclic ($T_{24\text{-}30}$) thermal regimens



Intervals from 6 h to 18 h correspond to the light phase (white) and from 18 h to 6 h to the dark phase of the day (dark gray). Values are the percentage of food ingested relative to the total offered during the experiments, and empty circles represent individual data. Different letters indicate statistical differences for Tukey's pairwise comparisons between groups or phases, at $p < 0.05$.

Bullfrogs did not exhibit changes in body mass during the time in the laboratory (Section 3.1). However, the body mass of frogs exposed to T_{24}, T_{30} and $T_{24\text{-}30}$ regimens pooled together shows a positive correlation with the rate of feeding success and the voluntary food intake (p = 0.0135 and p = 0.0271, respectively; Figure 10). This relationship reflects body mass differences among the experimental groups, with frogs of the T_{24} group being larger than frogs of the T_{30} and $T_{24\text{-}30}$ groups ($F = 53.95$, $R^2 = 0.5822$, and $p < 0.0001$) even though there was a tendency for higher feeding success in the T_{30} regimen compared to T_{24} (p = 0.0777).

Figure 10 – Feeding behavior as a function of body mass in *L. catesbeianus* frogs exposed to constant (T_{24}, T_{30}) or cyclic ($T_{24\text{-}30}$) thermal regimens

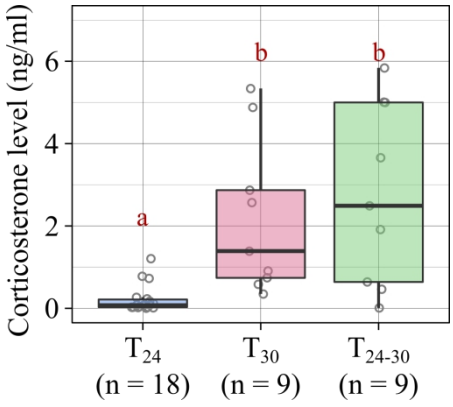


Each point represents one individual frog, and the solid lines describes a significant correlation for feeding success (A) and for food intake (B) for data of the T_{24}, T_{30} and $T_{24\text{-}20}$ regimens pooled together according to the equations $y = 0.1519 + 1.0118 \cdot x$ ($p = 0.0135$) and $y = 0.1569 + 1.0092 \cdot x$ ($p = 0.0271$), respectively.

3.5 Plasma corticosterone levels (CORT)

The exposure to different temperature regimens caused pronounced hormonal level changes, as shown in Figure 11. The levels of CORT were low, around 0.22 ng/ml in frogs exposed to T_{24}, and almost 900 % and 1150 % higher after exposure to T_{30} and $T_{24\text{-}30}$ ($p = 0.0007$ and 0.0084, respectively), with no statistical difference between T_{30} and $T_{24\text{-}30}$ ($p = 0.6859$). These results suggest that exposure to increased temperature in both constant and cyclic regimens stimulated a stress response in the frogs. The animals' handling time during blood collection did not affect on CORT levels ($p = 0.3616$) and was discarded during model selection.

Figure 11 – Plasma corticosterone levels in *L. catesbeianus* frogs exposed to constant (T_{24}, T_{30}) or cyclic ($T_{24\text{-}30}$) thermal regimens

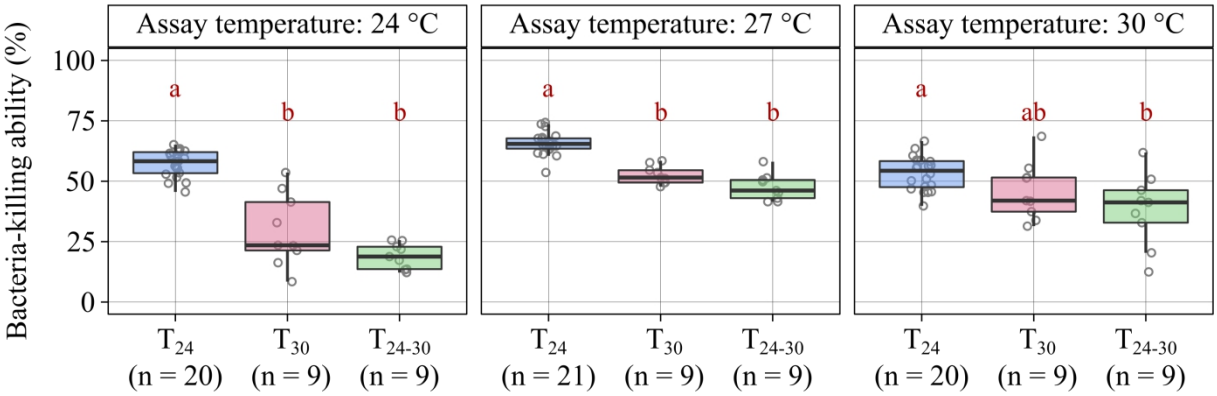


Empty circles represent the individual data. Different letters indicate statistical differences for Tukey's pairwise comparisons between groups, at $p < 0.05$.

3.6 Plasma bacteria-killing ability (BKA)

The exposure of frogs to increased temperature changed the general bacteria-killing ability of blood plasma, and this effect was complementarily modulated by the assay temperature (Figure 12). The BKA was lower in frogs exposed to T_{30} compared to T_{24} in assays performed at 24 °C ($p < 0.0001$) and 27 °C ($p = 0.0042$), as well as in frogs exposed to $T_{24\text{-}30}$ in assays at 24 °C ($p < 0.0001$), 27 °C ($p < 0.0001$), and 30 °C ($p = 0.0006$). Additionally, there was no difference between the BKA of frogs exposed to T_{30} and $T_{24\text{-}30}$ in assays performed at 24 °C ($p = 0.1427$), 27 °C ($p = 0.9241$), and 30 °C ($p = 0.5778$). Thus, these results suggest that exposure to 30 °C in both constant and cyclic regimens partially reduced the pathogen lysis immune response in bullfrogs.

Figure 12 – Plasma bacteria-killing ability of *L. catesbeianus* frogs exposed to constant (T_{24}, T_{30}) and cyclic ($T_{24\text{-}30}$) thermal regimens



Empty circles represent the individual data. Different letters indicate statistical differences for Tukey's pairwise comparisons between groups, at $p < 0.05$.

Discussion

4.1 The experimental system

In most laboratory studies, adult bullfrogs were housed in terrariums or plastic boxes with a shallow water area (*e. g.* FIGUEIREDO *et al.*, 2021 and GUEVARA-MOLINA; GOMES; CAMACHO, 2020). Although this method does not allow submersion or locomotion in the water, Pough (1991) pointed out the importance of habitat diversity to the care of experimental amphibians, and a wide variety of cages for aquatic frogs that incorporate this requirement can also be found in literature. For example, Bardsley and Harmsen (1972) suggested a flow-through system of compartmentalized cages. Alworth and Vazquez (2009) suggested a protocol specifically for *Lithobates catesbeianus* juvenile and adults, in which frogs are individually housed inside buckets with access to both water and a dry perch. Our system is a modified version of Alworth and Vazquez's protocol, greatly improved by the addition of a flat dry and easily accessible surface that provides an underwater shelter, besides water recirculation, filtration, and temperature control (Section 2.2).

The temperature curves obtained for the T_{24}, T_{30} e $T_{24\text{-}30}$ regimens (Table 1; Figure 3) indicate that the experimental system performed generally well and was suitable for the study. One point to be highlighted is the slow rate of water cooling in the $T_{24\text{-}30}$ regimen, and as a consequence, frogs in the cyclic regimen were exposed to elevated temperatures during a considerable part of the daily cycle, whereas they spent less time in the lower temperature range.

4.2 Basic behaviors expressed in the experimental system

Frogs maintained individually in the experimental system performed few transitions (no more than 50 transitions per day) between microhabitats throughout the 24 h period, and therefore, the estimates of daily locomotor activity were generally low (Figure 7A, grey boxes). This result is in accordance with previous observations on post-metamorphic bullfrogs by Arruda and colegues (2014), that used a two-floors system with increased microhabitat diversity, and the predominant behavior during the 24 h period was inactivity in water and on dry ground. However, contrary to what we expected, the daily amount of transitions did not change significantly with heat exposure either in T_{30} or $T_{24\text{-}30}$ regimen (Figure 7A), suggesting that kinetic effects of temperature on metabolic rates might have increased energy expenditure without an increase in locomotor activity (JIMENO; HAU; VERHULST, 2018).

The bullfrogs spent only a small fraction (less than one hour) of the daily time completely submerged, and alternated between partial and total emersion on the flat surface almost all the time (means of 59 % and 38 % of the day, respectively) in the T_{24} regimen, for instance (Figure 8A). The temperature rise in the T_{30} regimen significantly increased by 72 % the daily time that frogs spent emerged on the flat surface, but not in the $T_{24\text{-}30}$ regimen (Figure 8A). This result indicate that chronic exposure to water at constant 30 °C induced an escape behavior from the aversive stimulus and the search for a colder microhabitat (HUEY; KINGSOLVER, 2019). The air temperature near the flat surface was around 2 °C below water temperature in the T_{30} regimen (Figure 3). Additionally, a parallel study using agar models found simulated body temperatures around 1 °C below water temperatures when models were totally emerged (Lidia S. YANO, unpublished results). Therefore, the increase of air exposure time on the surface may have offered some thermal relief in the T_{30} regimen.

During high air temperature exposure, physiological responses are triggered to decrease evaporative water loss, such as decrease in cutaneous gas exchange, drying and stiffening of the integument, increased mucus discharge, lower rates of water loss, and reduced efficiency of evaporative cooling (GUEVARA-MOLINA; GOMES; CAMACHO, 2020; LILLYWHITE, 1971). However, in the present study the air in the buckets was saturated with water vapor, eliminating risks of desiccation in frogs exposed to air on the flat surface. On the other hand, immersion in water at 30 °C might have direct effects on skin defenses and respiratory gas exchange, favoring the choice for water emergence. First, because warmer water can damage the skin mucous physical barrier, or influence the chemical antimicrobial peptides barrier, and these effects may favor microbial colonization and impair the skin innate immune defenses (PELLI *et al.*, 2010; ROBAK; RICHARDS-ZAWACKI, 2018; VARGA; BUI-MARINOS; KATZENBACK, 2018). Second, because warmer water generally has lower concentrations of dissolved oxygen, which hinders the cutaneous gas exchange and favors the behavior of water emergence and air breathing, even though the high levels of air relative humidity inside buckets may reduce the efficiency of oxygen uptake by pulmonary respiration (BLAUSTEIN *et al.*, 2010; PÖRTNER; BOCK; MARK, 2017). Despite this, the prevalence of the emergence behavior in frogs exposed to 30 °C in the constant regimen might be advantageous by favoring oxygen uptake from air and sustaining metabolism and performance.

4.3 Temporal organization under constant and cyclic temperature regimens

In the present study, we expected that the use of a daily temperature cycle would enhance day-night organization of behavior and physiology in ectothermic anurans. In chronobiological research, activity is one major indicator of this temporal organization (ASCHOFF, 1971), and the absence of a day-night pattern of locomotor activity in the T_{24} regimen (Figure 7) would be an indication that the bullfrogs are arrhythmic (ARRUDA *et al.*, 2014; HADFIELD; CLAYTON; BARNETT, 2006; KRUPA, 2002). However, in contrast to arrythmicity at constant 24 °C, bullfrogs under T_{30} and $T_{24\text{-}30}$ regimens displayed significant day-night activity organization. In the cyclic regimen, the locomotor activity concentrated mostly on the heating phase of the temperature cycle (Figure 7B), further suggesting the acute effects of higher temperature in enhancing rhythmicity. According to the video analysis, frogs in the $T_{24\text{-}30}$ regimen stayed longer in the water during the heating phase, transitioning many times between total and partial submersion for lung ventilation (GARGAGLIONI; MILSOM, 2007; See the Appendix B for detailed frogs' microhabitat choice). Although this day-night pattern was significant, however, short bouts of locomotor activity were spread throughout the 24 hours showing that daily rhythmicity in bullfrogs and perhaps in anurans in general are not as robust (ADLER, 1971) as in other vertebrates. In an essay about activity patterns in mammals, Hazlerigg and Tyler (2019) discussed that the temporal control of activity varies widely across the taxa, and that the absence of a strong circadian organization of activity does not imply absence of strong temporal organization of other behaviors and of physiology in general. Therefore, in ectothermic animals, such as anurans with sit-and-wait strategies and no robust day-night activity pattern, measuring more than one behavior to map circadian rhythms might be essential.

Other behavioral aspects of the bullfrogs were analyzed in search for more proxies of their internal temporal organization. However, we found no effect of the phase of the day over the time that frogs spent emerged under the T_{30} and the $T_{24\text{-}30}$ regimen, either from 6 h to 12 h during water heating or from 12 h to 18h when the water temperature was stable at 30 °C (Figure 8B). As a consequence, the behavioral choice of microhabitat also showed no robust day-night pattern. Otherwise, previous study by Arruda *et al*. (2014) found that bullfrogs' movement on dry ground was more intense during the day at temperatures around 29 °C than during the night, at temperatures around 24 °C. Given the considerable differences of protocol associated with these contrasting results, more studies would contribute to clarify whether there is a temporal basis underlying the microhabitat preference in bullfrogs.

The feeding behavior, described by means of feeding success and voluntary food intake, presented slightly higher values in the beginning of the night, from 18 h to 24 h, in frogs under the T_{24} regimen but not when exposed to heat in the other two regimens (Figure 9). In contrast, Casali (2010) and Arruda *et al*. (2014) found that post metamorphic bullfrogs under fluctuating temperatures are more likely to ingest food during dawn. Adding methodological differences, the behavioral pattern of juvenile bullfrogs may contrast with that of post metamorphic frogs, as well as with recent evidence of nocturnal traces in adult bullfrogs found in field studies on ecology and calling behavior, and in laboratory studies on hormonal daily variation (HÖDL; AMÉZQUITA, 2001; LAUFER *et al.*, 2017; TITON *et al*., 2021). Rusak (1981) pointed out that amphibians may undergo developmental changes of behavioral rhythmicity, as salamanders seem to be diurnal in the larval stage but nocturnal as adults, and juvenile toads can be less strictly nocturnal than the adults. Together with the absence of temporal differences in the other two regimens (Figure 9), we conclude that feeding behavior too is poorly rhythmic in bullfrogs and may be highly flexible in time under heat exposure, and possibly in face of other environmental challenges.

4.4 Stress levels and immune capacity

Associations between arrhythmic individuals and shorter lifespan in a population (HURD *et al.*, 1998; KUMAR ; MOHAN; SHARMA, 2005), for instance, have strengthened the importance of internal temporal organization for general health (DAAN; ASCHOFF, 1982). In this context, it is intriguing that $T_{24\text{-}30}$ and T_{30} bullfrogs, which presented weak signs of temporal organization, were associated with higher CORT levels than the T_{24} group, suggesting an overriding effect of heat exposure. The glucocorticoid hormone, a biomarker of stress response, acts collectively with a large network of neurohormones to maintain the organisms' homeostasis (NARAYAN *et al*., 2019). Under the T_{24} regimen, CORT levels are close to zero in plasma collected during the day at 13 h (Figure 11), which is in line with the expected range for this time measured in a more thorough hormone investigation in adult bullfrogs (TITON *et al*., 2021). Although the isolated information of low CORT levels does not directly mean that stressors are absent, frogs under T_{24} in the present study were exposed to a temperature close to that experienced in the farm and low CORT can be taken as an indication of physiological acclimation to laboratory conditions. Moreover, CORT levels were increased by ten times in plasma collected at 13 h from frogs under T_{30} and $T_{24\text{-}30}$, suggesting stress and homeostasis disturbance during exposure to higher temperatures. However, CORT increases of similar order of magnitude were found during the dark phase in

bullfrogs kept at 21 °C (TITON *et al.*, 2021). Thus, given that glucocorticoids have many downstream effects on energy expenditure, this increased CORT could be also interpreted as adjustments of functions to variation in metabolic demands (JIMENO; HAU; VERHULST, 2018). For instance, a parallel study in our laboratory found that glycogen levels were reduced in muscle and increased in liver of bullfrogs under T_{30} and $T_{24\text{-}30}$ (Lidia S. YANO, unpublished results), suggesting that increased CORT during the light phase in both regimens may be associated with metabolic reorganization in the face of increased temperature.

The BKA values with plasma of frogs under the T_{24} regimen are also in good agreement with those reported for adult bullfrogs by Titon *et al.* (2021). This result attests good assay reproducibility and that the immune humoral response of frogs in the present study was preserved. Additionally, the lower BKA in the T_{30} and $T_{24\text{-}30}$ regimens can be attributed to the high temperature rather than to housing conditions or feeding and checking routines in the laboratory (Section 4.1). Moreover, the combination of higher CORT levels and lower BKA gives support to the idea that frogs were under thermal stress in both regimens (Figures 11 and 12). It has been shown that the exposure to long-term stress can suppress or dysregulate innate and adaptive immune responses by suppressing numbers, trafficking, and function of immune protective cells, by inducing low-grade chronic inflammation, or by altering cytokine balance, ultimately supressing protective immune responses or exacerbating pathological immune responses (DHABHAR, 2014). While corticosteroid hormones play an important role in the mobilization of energy reserves in acute stress in amphibians, for example through elevation of glucose levels, in chronic or extreme stress they may impair amphibian immune defenses through loss of lymphocyte or diminished antimicrobial peptides (ROLLINS-SMITH, 2017). The study of Lima *et al*. (2020) on bullfrogs showed that BKA's optimal thermal range after 16 days of exposure to a 28 °C constant air temperature regimen were between 18 °C and 29 °C, with mean value around 22 °C, and temperatures above 29 °C exceed the BKA upper optimal limit. In their laboratory conditions, the authors found CORT levels around 2 to 4 ng/ml, which are similar to the CORT levels of frogs exposed to the T_{30} and the $T_{24\text{-}30}$ regimens in the present study, and they concluded that the exposure to high temperatures might decrease the immune function in bullfrogs due to chronic stress response. The combination of increased CORT levels and decreased BKA ability in amphibians was also observed in natural contexts of stress such as during long- or short-distance migration, and during confinement (ROLLINS-SMITH, 2017).

Besides CORT and BKA levels, different bioenergetic markers can be used to distinguish moderate stress, which is compatible with long-term survival of a population,

from extreme stress (SOKOLOVA, 2013). According to this author, the tolerance to stress is linked to the energy metabolism capacity to aerobically support basal maintenance, activity, maturation, growth and deposition of energy reserves. Energy-limited populations are seen when there is a pejus range of environmental conditions, leading to suboptimal growth and reproductive rates, or a pessimum range, when the aerobic scope of the organism is not enough to support the basal metabolism and partial anaerobiosis sets in (SOKOLOVA, 2013). Accordingly, the activity of citrate synthase, a marker of aerobic metabolism, is lower in heart and liver tissue of the bullfrogs exposed to the T_{30} and $T_{24\text{-}30}$ regimens (Lidia S. YANO, unpublished results). This finding suggests that the maximum temperature of 30 °C possibly lies in the pejus range. Additionally, frogs in the cyclic regimen were exposed to elevated temperatures during a considerable part of the daily cycle (Section 4.1), and the similarity between the effects of high temperature in the cyclic and constant regimens could be explained at least in part by heat stress for a lasting period in the cyclic regimen. Altogether, we may conclude that severe impairment of function and performance would possibly result of a more prolonged exposure of bullfrogs to 30 °C in either regimen.

Conclusions

This study proposes an effective way of controlling water temperature in experiments with recirculating water that allowed the investigation of both the temporal organization and the impact of increased temperature on different physiological levels of *L. catesbeianus* juvenile frogs, while stimulating the look at animal welfare in the individual housing of anurans in the laboratory by providing different microhabitats to the animals.

The bullfrogs showed low locomotor activity and explored the microhabitats of total emersion and partial submersion in water more markedly than the total submersion. But the basic behaviors of feeding, locomotor activity and microhabitat choice expressed in the experimental system were poorly rhythmic under the T_{24} regimen, and more studies would contribute to clarify whether there is a temporal basis underlying the behavioral activity in bullfrogs. Under the 30 °C exposure, the frogs altered their day-night pattern of locomotor activity and feeding behavior in the T_{30} and the $T_{24\text{-}30}$ regimen. All these results suggest that their behavior are highly flexible in time in face of environmental challenges. The kinetic effects of temperature on metabolic rates might have increased energy expenditure, without an increase in locomotor activity, at the same time that the animals increased their preference for staying out of the water, possibly preserving skin innate immune defenses and favoring oxygen uptake from the air, sustaining metabolism and performance, especially under the T_{30} regimen.

The increased temperature triggered homeostasis disturbance in frogs exposed to both T_{30} and $T_{24\text{-}30}$ regimen, with higher CORT levels and lower BKA levels, possibly associated with metabolic reorganization and dysregulation of innate immune responses in face of thermal stress. The temperature of 30 °C possibly lies on a pejus range, impairing the aerobical capacity of the energy metabolism to sustain basal maintenance, activity, maturation, growth, and deposition of energy reserves. Because the frogs in the $T_{24\text{-}30}$ regimen experienced a asymmetric exposure to 30 °C, longer than to lower temperatures, our results suggest that severe impairment of function and performance would possibly result of a more prolonged exposure of bullfrogs to 30 °C in either regimen. Future studies that better resemble temperature variation in natural or seminatural conditions would contribute to investigate the impacts of cyclic increased temperature.

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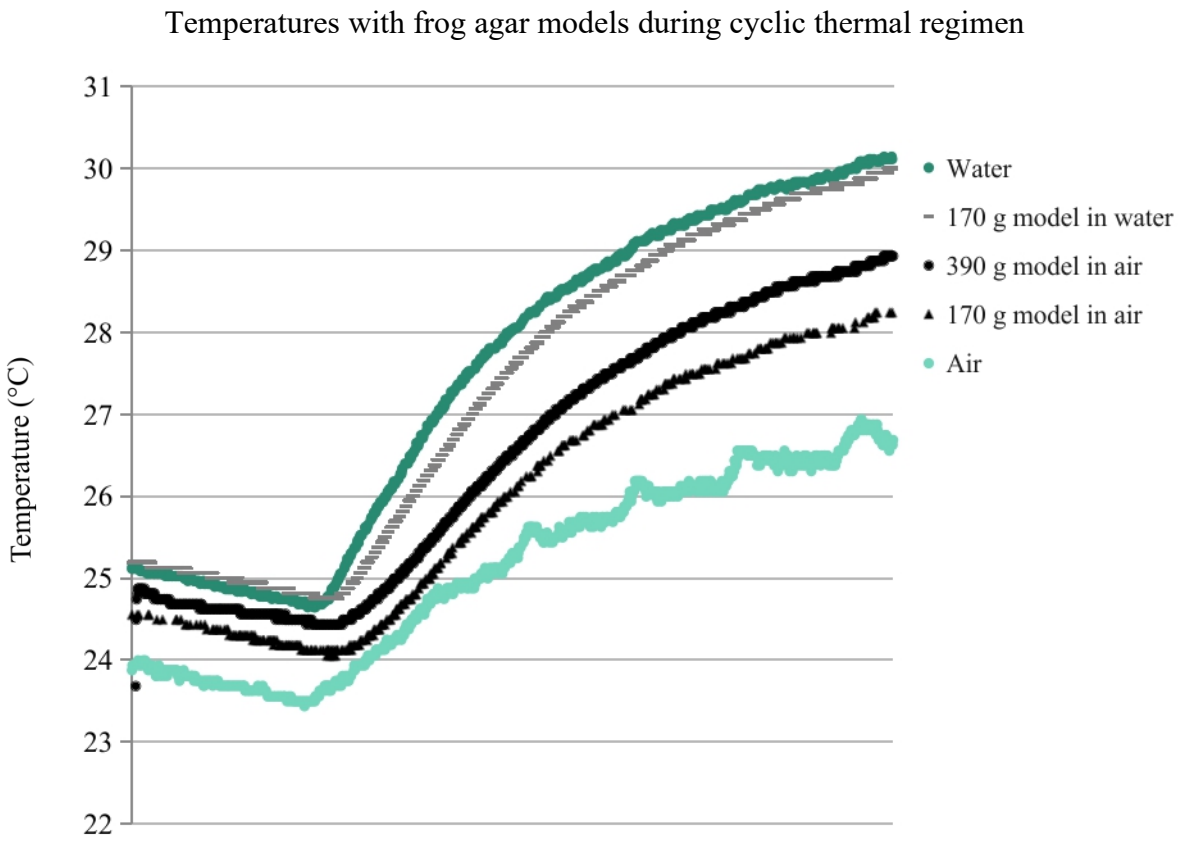
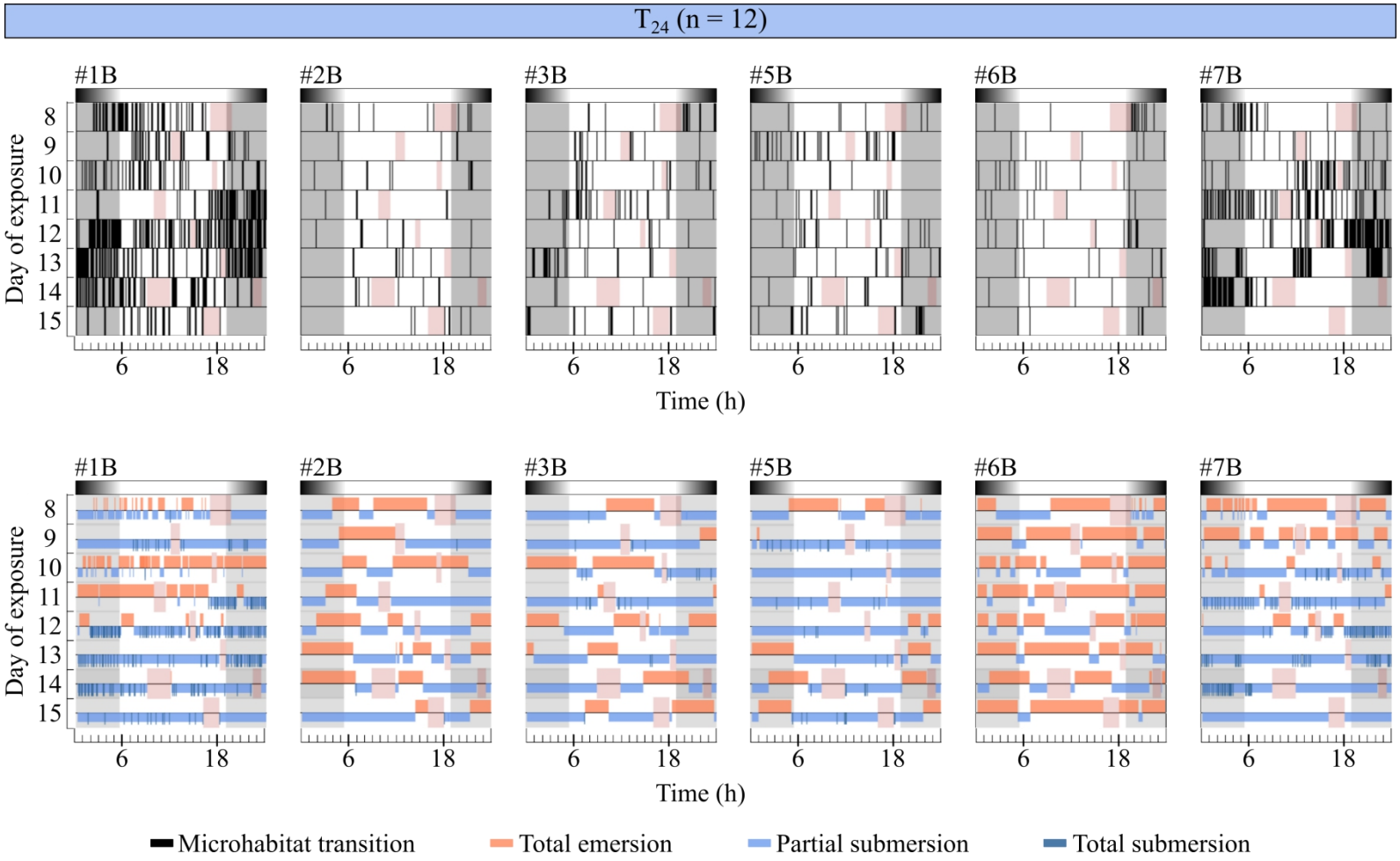
Appendix

Appendix A – Measures with agar model in the experimental system



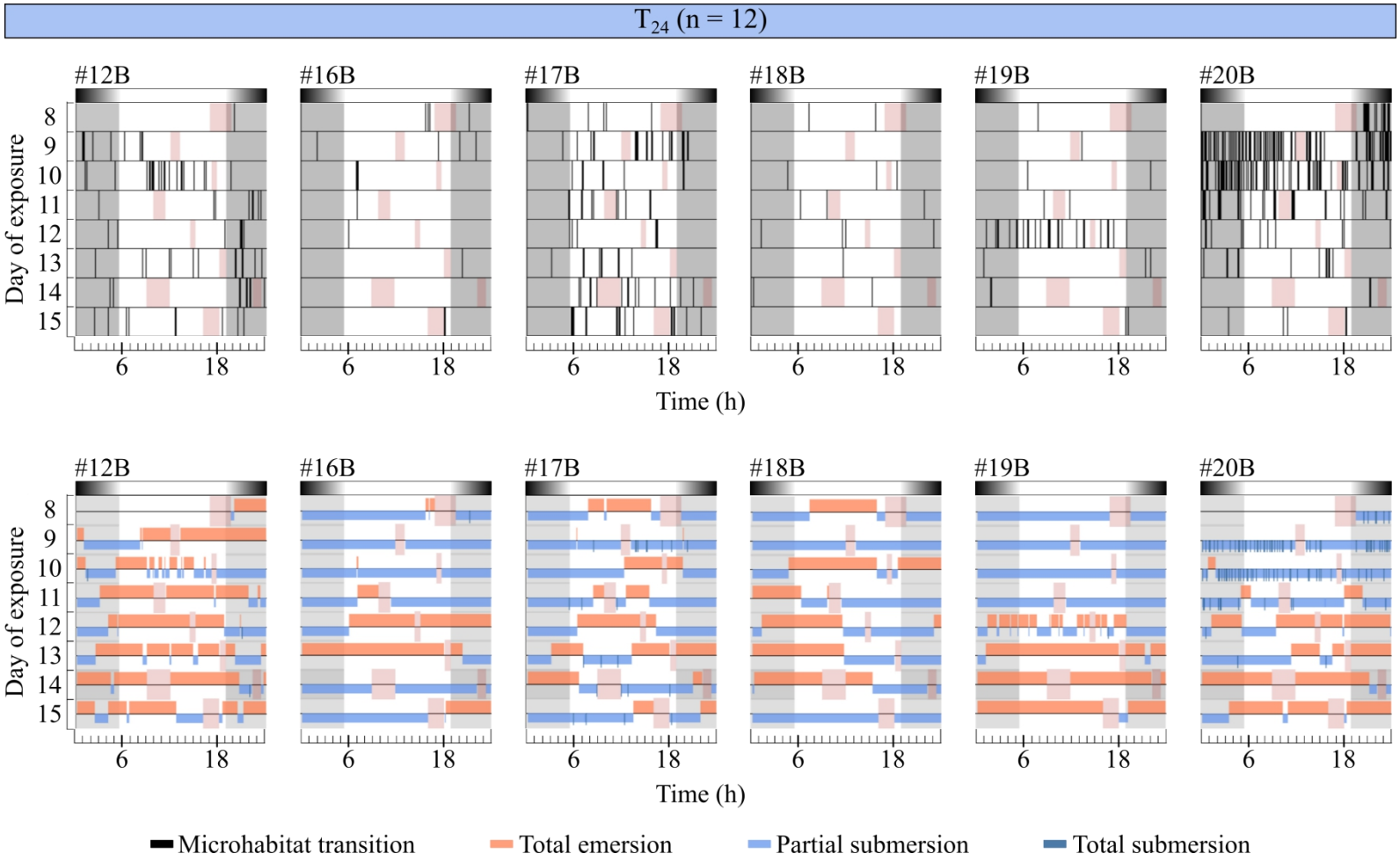
Frog agar models with mass ranging from 170 g to 390 g were used to estimates *L. catesbeianus* frogs body temperatures when exploring different microhabitats in the experimental system. The cyclic $T_{24\text{-}30}$ thermal regimen was used to provide estimates of body temperature variation in all temperatures experienced by the animals throughout nine hours of measurements. The two sizes of agar models can be used to infer the body temperature of the bullfrogs with body mass contained in the described interval. The models' temperature varied similarly to the water temperature itself (dark green) when positioned inside the water (gray traces). On the other hand, in the microhabitat of total emmersion, the models' reached intermediate temperature values (black) between the water and the air (light green).

Appendix B – Actograms and microhabitat choice under T_{24}, T_{30}, and $T_{24\text{-}30}$ regimens

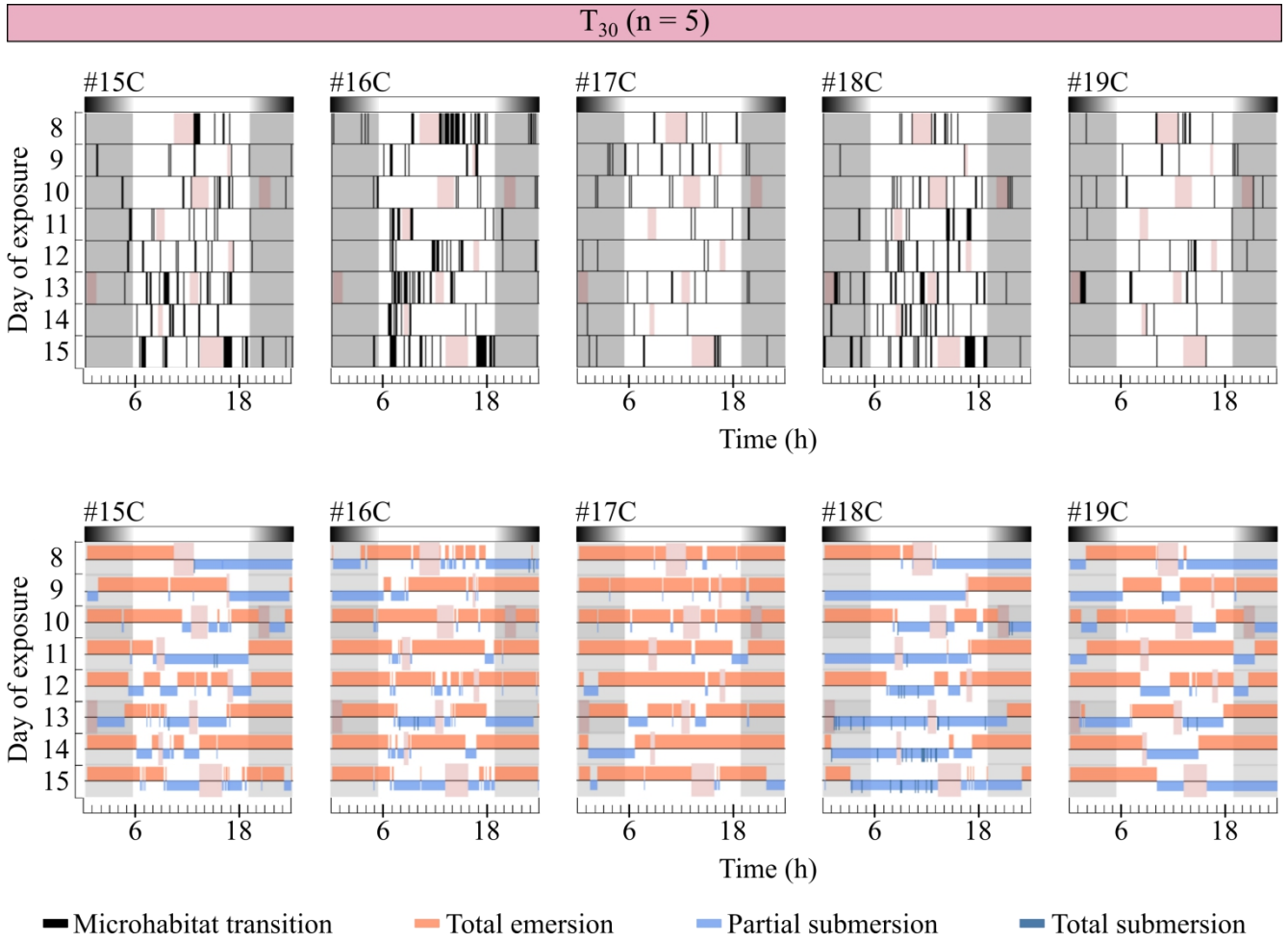


The first row marks the microhabitat transition (black) of each frog between the 8th and the 15th day of exposure to the termal regimen, taken as a proxy of the frogs' locomotor activity. The second row shows the animals' respective length of stay at each microhabitat, taken as a proxy of microhabitat choice: total emersion in air (orange), partial submersion in water (light blue) and total submersion in water (dark blue). Pink areas indicate the human presence during feeding and checking routines, and were disregarded in the analysis. The bars on the top of the plots indicate the natural photoperiod variation, and the night phase (gray background) of the light-dark cycle coincides with the cooling phase of the $T_{24\text{-}30}$ regimen (page 59).

Appendix

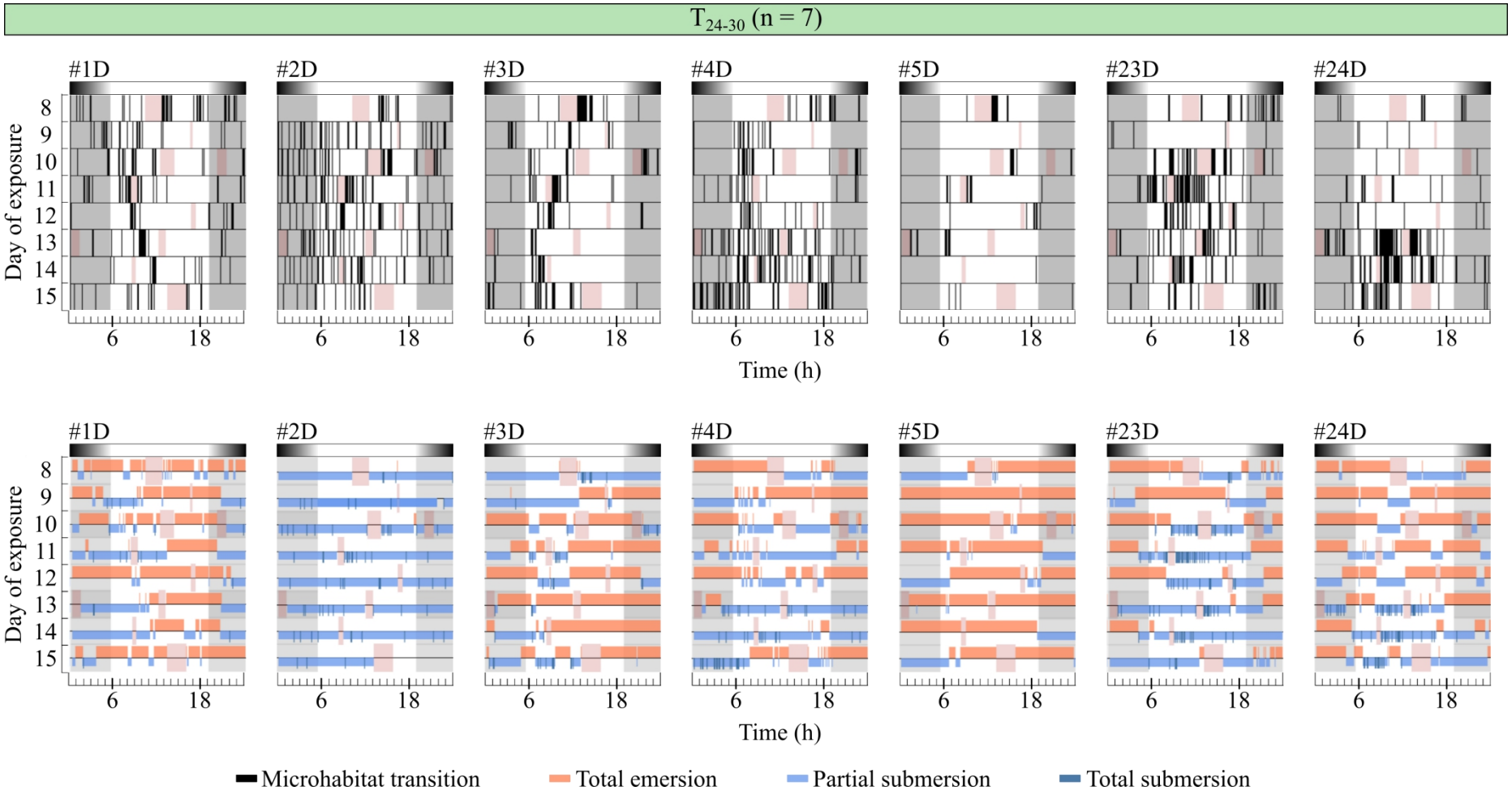


The first row marks the microhabitat transition (black) of each frog between the 8th and the 15th day of exposure to the termal regimen, taken as a proxy of the frogs' locomotor activity. The second row shows the animals' respective length of stay at each microhabitat, taken as a proxy of microhabitat choice: total emersion in air (orange), partial submersion in water (light blue) and total submersion in water (dark blue). Pink areas indicate the human presence during feeding and checking routines, and were disregarded in the analysis. The bars on the top of the plots indicate the natural photoperiod variation, and the night phase (gray background) of the light-dark cycle coincides with the cooling phase of the $T_{24\text{-}30}$ regimen (page 59).



The first row marks the microhabitat transition (black) of each frog between the 8th and the 15th day of exposure to the termal regimen, taken as a proxy of the frogs' locomotor activity. The second row shows the animals' respective length of stay at each microhabitat, taken as a proxy of microhabitat choice: total emersion in air (orange), partial submersion in water (light blue) and total submersion in water (dark blue). Pink areas indicate the human presence during feeding and checking routines, and were disregarded in the analysis. The bars on the top of the plots indicate the natural photoperiod variation, and the night phase (gray background) of the light-dark cycle coincides with the cooling phase of the $T_{24\text{-}30}$ regimen (page 59).

Appendix

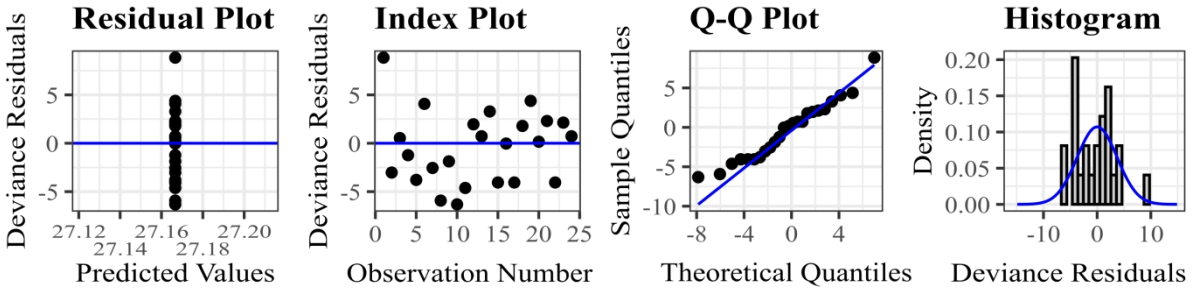


The first row marks the microhabitat transition (black) of each frog between the 8th and the 15th day of exposure to the termal regimen, taken as a proxy of the frogs' locomotor activity. The second row shows the animals' respective length of stay at each microhabitat, taken as a proxy of microhabitat choice: total emersion in air (orange), partial submersion in water (light blue) and total submersion in water (dark blue). Pink areas indicate the human presence during feeding and checking routines, and were disregarded in the analysis. The bars on the top of the plots indicate the natural photoperiod variation, and the night phase (gray background) of the light-dark cycle coincides with the cooling phase of the $T_{24\text{-}30}$ regimen.

Appendix C – Formulas and residual plots of the minimal adequate models

Locomotor activity

Figure 7A – Minimal adequate model	Type	Family
24h Activity ~ 1	GLM	Quasipoisson



12h Activity ~ Treatment + Phase + (1|Animal)	GLMM	Poisson

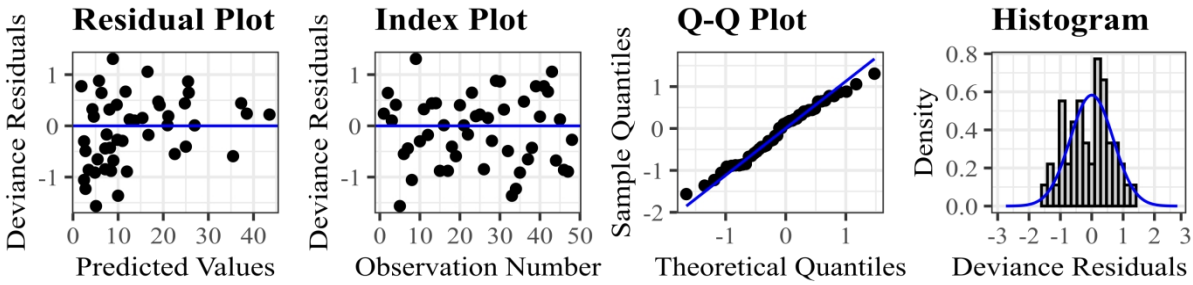
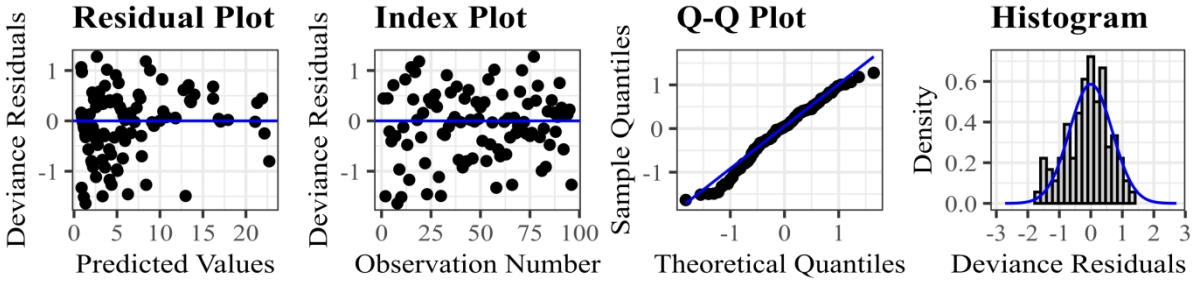
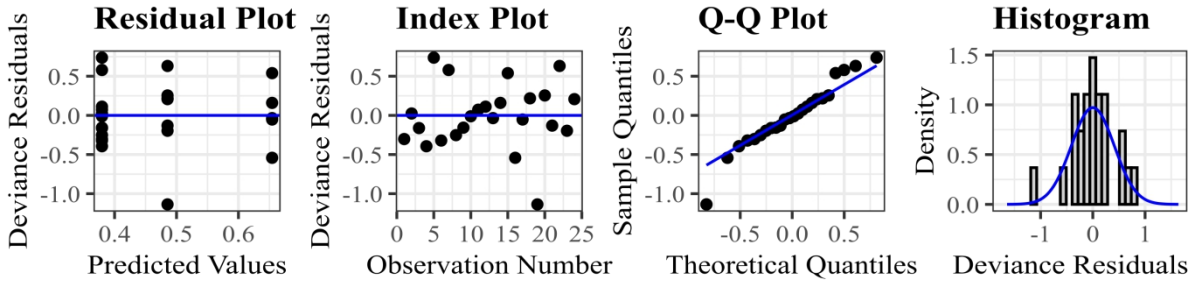


Figure 7B – Minimal adequate model	Type	Family
*6h Activity ~ Treatment * Phase + (1|Animal)*	GLMM	Poisson

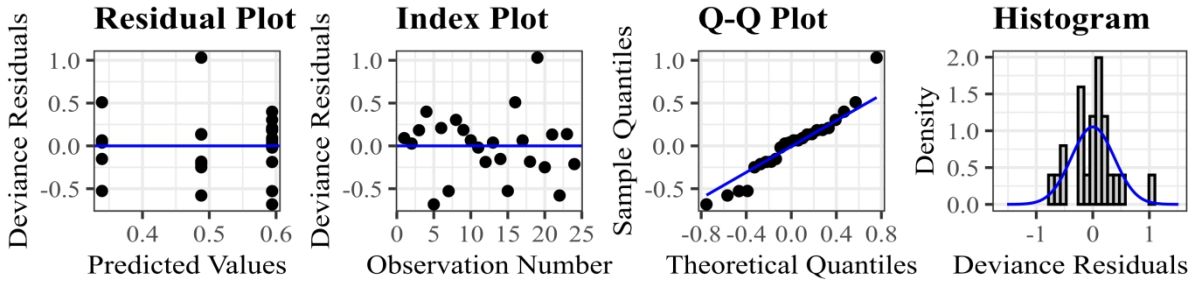


Microhabitat choice

Figure 8A – Minimal adequate model	Type	Family
24h Total emmersion ~ Treatment	GLM	Quasibinomial



24h Partial submersion ~ Treatment	GLM	Quasibinomial



24h Total submersion ~ 1	GLM	Quasibinomial

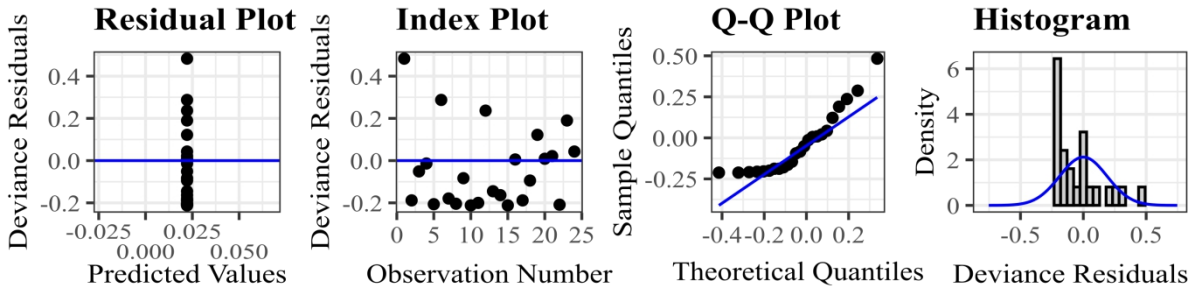


Figure 8B – Minimal adequate model (continue)	Type	Family
6h Total emmersion ~ Treatment + (1|Animal)	LMM	Normal

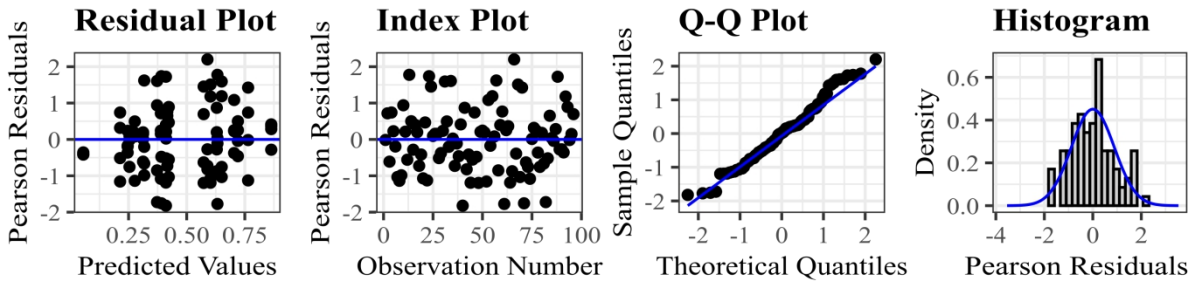
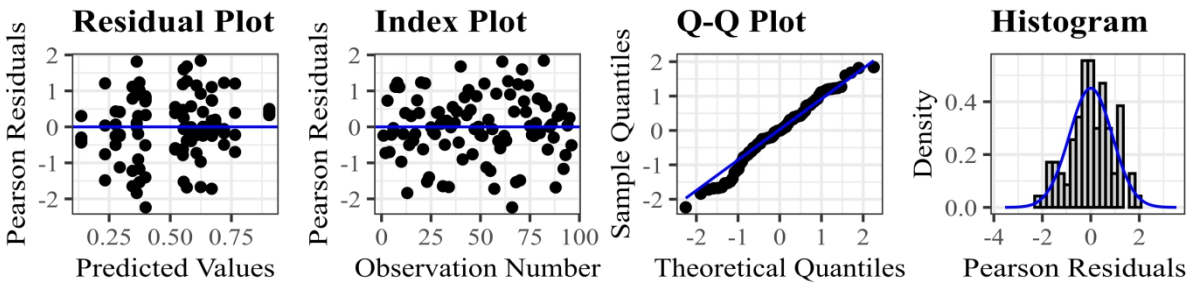
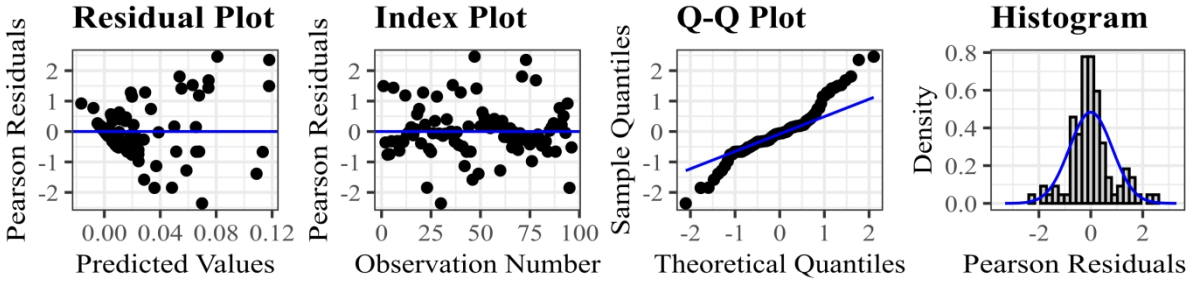


Figure 8B – Minimal adequate model (continuation)	Type	Family
6h Partial submersion ~ Treatment + (1|Animal)	LMM	Normal

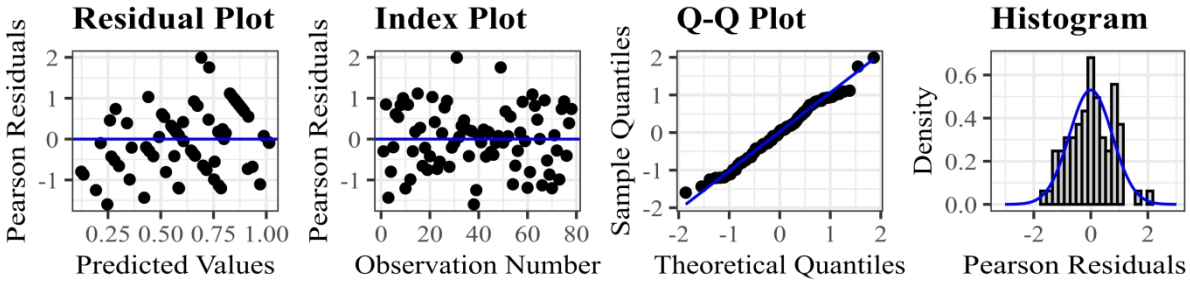


*6h Total submersion ~ Treatment * Phase + (1|Animal)*	LMM	Normal



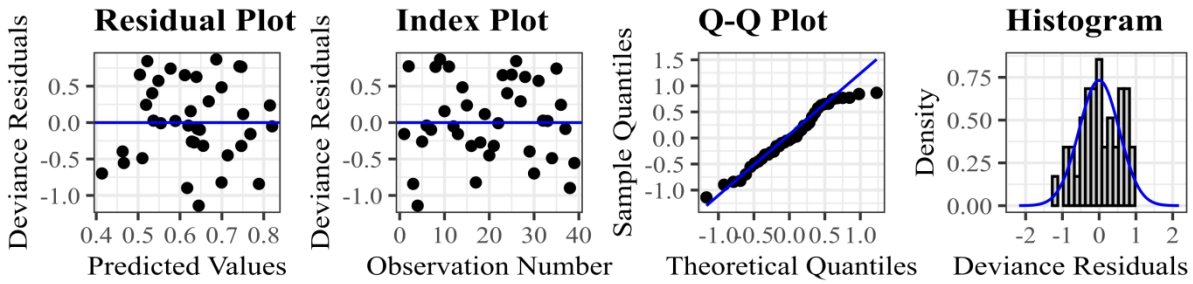
Feeding behavior

Figure 9 – Minimal adequate model	Type	Family
*12h F. success ~ Body mass + Treatment * Phase + (1|Animal)*	LMM	Normal

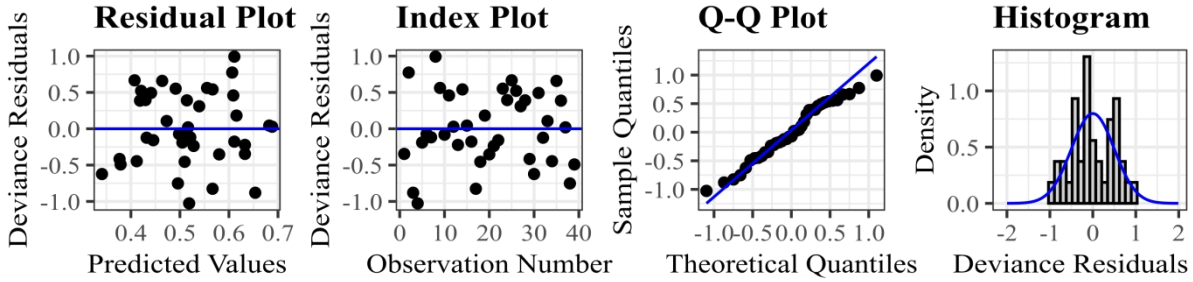


Appendix

Figure 10 – Minimal adequate model	Type	Family
24h Feeding success ~ Body mass	GLM	Quasibinomial

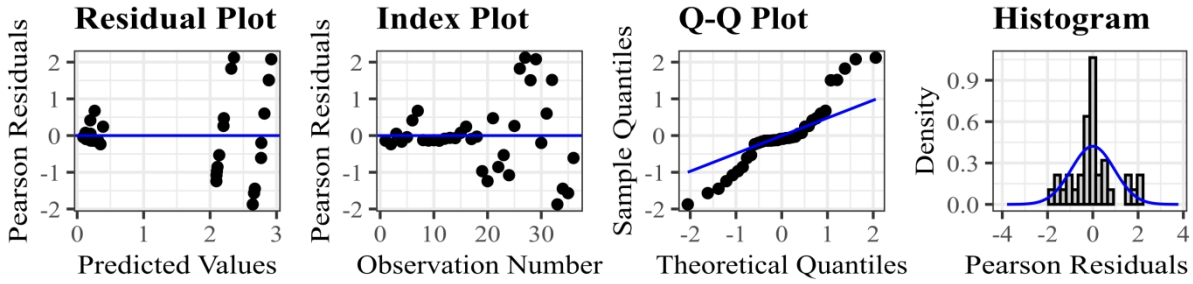


24h Voluntary food intake ~ Body mass	GLM	Quasibinomial



CORT

Figure 11 – Minimal adequate model	Type	Family
CORT ~ Treatment + (1|Position)	LMM	Normal



BKA

Figure 12 – Minimal adequate model	Type	Family
*BKA ~Treatment * Assay Temperature + (1|Animal)*	LMM	Normal