Gustavo Adolfo Agudelo Cantero

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Thesis submitted to the Institute of Biosciences of the University of São Paulo, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Area of General Physiology.

Supervisor: Carlos A. Navas
Co-supervisor: Fernando R. Gomes

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Dedication

To my loving and faithful God and Lord,
my reason, inspiration, and motivation.

To my beloved Natalia,
my sweet love and loyal companion.

To Gustavo, Luzmila, César & Adriana,
my loving and supportive parents.

To Jennifer, Iván, & Valentina,
my beloved siblings.

To Nathali, my most cheerful cousin.
This thesis is for both of us!

Epigraph

“*I want to know God’s thoughts…*”

Albert Einstein (1879-1955)

“*I was merely thinking God’s thoughts after Him…*”

Johannes Kepler (1571-1630)

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Table of Contents

Abstract

Climate change (CC) is altering the magnitude, frequency, and predictability of extreme events, modifying historical averages throughout the globe. However, the study of biological responses to CC have prioritized effects of altered mean climatic conditions, which are rarely experienced by organisms. Then, our comprehension of organismal responses to climatic variation remains limited. This Doctoral thesis contributes to fill that gap by studying the physiological and behavioral responses of small ectothermic animals to thermal variations in space and time. Results included here answered three questions: (1) How much microclimatic thermal diversity usable by anuran larvae may exist across aquatic microhabitats at a local scale? (2) Does local microclimatic thermal diversity promote interspecific variation in CT_{max} of anuran larvae at the same spatial scale? (3) What behavioral strategies are employed by fruit flies (*Drosophila melanogaster*) when navigating through extreme thermal landscapes? We found that habitat complexity generated high microclimatic thermal diversity usable for anuran larvae within a small patch of the Brazilian Atlantic Forest. Specifically, water bodies filtered differently thermal variations at the local environment given differences in their structural characteristics (e.g., temporality, water motion) and associated canopy cover. The latter, in particular, was a very important modulator of microclimatic diversity, for water bodies inside the forest were in general less thermally variable relative to counterparts in open areas. In addition, larvae of an anuran assemblage that inhabits our study area displayed high CT_{max} diversity, and species variation paralleled microclimatic exposure more than phylogenetic relatedness. In other words, species were more similar in their CT_{max} according to where larvae develop and not how close they are in phylogeny. Moreover, the most heat tolerant species in the assemblage were those inhabiting the warmest water bodies in open areas, and for that reason are also the most vulnerable to further warming. On the other hand, fruit flies displayed diverse behavioral responses while navigating through extreme thermal landscapes, beyond simply thermophobia. Behavioral strategies varied at the population level and displayed distinct evolutionary potential when expressed at an extremely hot or cold thermal landscapes. Collectively, results included in this thesis showed that spatial and temporal thermal variations at scales relevant to organisms favor physiological and behavioral diversity across species and within populations. Understanding such diversity, what promotes it, and its vulnerability to CC, is crucial to safeguard the future of life in the Anthropocene.

Resumo

As mudanças climáticas (MC) estão alterando a magnitude, frequência e previsibilidade de eventos extremos, modificando médias históricas em todo o mundo. No entanto, o estudo das respostas biológicas às MC tem priorizado os efeitos das alterações nas condições climáticas médias, as quais são raramente experimentadas pelos organismos. Então, nossa compreensão das respostas dos organismos à variação climática permanece limitada. Esta tese de Doutorado contribui para preenchermos essa lacuna do conhecimento ao estudar as respostas fisiológicas e comportamentais usadas por pequenos organismos ectotérmicos a variações térmicas no espaço e no tempo. Os resultados aqui incluídos responderam três perguntas: (1) Quanta diversidade térmica microclimática utilizável por larvas de anuros pode existir em microhabitats aquáticos em escala local? (2) A diversidade térmica microclimática em escala local promove variação interespecífica no CT_{max} de larvas de anuros na mesma escala espacial? (3) Que estratégias comportamentais são usadas mosca-das-frutas (*Drosophila melanogaster*) ao navegar por paisagens térmicas extremas? Encontramos que a complexidade do habitat gerou alta diversidade térmica microclimática utilizável para larvas de anuros dentro de um pequeno pedaço da Mata Atlântica Brasileira. Especificamente, os corpos d'água filtraram variações térmicas locais de maneira diferente, dadas diferenças em suas características estruturais (p.ex., temporalidade, movimento da água) e cobertura de dossel associada. Este último fator, em particular, foi um modulador muito importante da diversidade microclimática, porque os corpos d'água dentro da floresta foram em geral menos variáveis termicamente em relação a contrapartes em áreas abertas. Além disso, as larvas de uma assembléia de anuros que habitam nossa área de estudo exibiram alta diversidade de CT_{max}, e a variação interespecífica refletiu à exposição microclimática mais do que as relações filogenéticas. Ou seja, as espécies foram mais semelhantes em seu CT_{max} de acordo com onde as larvas se desenvolvem e não quão próximas as espécies estão na filogenia. As espécies mais tolerantes ao calor foram aquelas que habitam os corpos d'água mais quentes em áreas abertas e, por esse motivo, também são as mais vulneráveis ao aquecimento. Por outro lado, as mosca-das-frutas exibiram diversas respostas comportamentais quando navegaram em paisagens térmicas extremas, e não apenas termofobia. As estratégias comportamentais variaram no nível da população e exibiram potencial evolutivo distinto quando expresso em paisagens térmicas extremamente quentes ou frias. Coletivamente, os resultados incluídos nesta tese mostram que variações térmicas espaciais e temporais em escalas relevantes para os organismos favorecem a diversidade fisiológica e comportamental entre as espécies e dentro das populações. Compreender essa diversidade, o que a promove, e sua vulnerabilidade às MC, é crucial para proteger o futuro da vida no Antropoceno.

General Introduction

1. *The problem*

The ongoing change on the Earth's climate system is an unequivocal and global, yet regionally heterogeneous, phenomenon that involves both natural and anthropogenic causes (IPCC, 2021). That climate change (CC) has consequences on biodiversity is also unquestionable (PÖRTNER *et al.*, 2021; WALTHER *et al.*, 2002). First-discovered fingerprints of CC on biodiversity included species range and phenological shifts (PARMESAN, 2006; PARMESAN; YOHE, 2003; ROOT *et al.*, 2003), but concerns of potential climate-driven extinctions of entire species was raised even before (POUNDS; CRUMP, 1994; POUNDS; FOGDEN; CAMPBELL, 1999). Nowadays, it is clear that CC impacts on biodiversity are evident at all levels of biological organization (MCCARTY, 2001; SEARS; ANGILLETTA, 2011; WALTHER *et al.*, 2002), and scale up from individuals to populations, communities, and ecosystems (MCCARTY, 2001; SCHEFFERS *et al.*, 2016). Some biological responses to CC are: individual body size reduction (SHERIDAN; BICKFORD, 2011); disrupted structure and dynamics of populations (LAUGHTON; KNELL, 2019; ZYLSTRA *et al.*, 2021), including declines and local extinction (SINERVO *et al.*, 2010; WIENS, 2016); altered biotic interactions (BIRKEMOE *et al.*, 2016; HARRINGTON; WOIWOD; SPARKS, 1999), structure (TANENTZAP *et al.*, 2020), and collapse of communities (IKNAYAN; BEISSINGER, 2018); and transformation of ecosystems (SPEED *et al.*, 2021). However, given the intricate integration among levels of biological organization, the causal linkages behind most of these patterns remain elusive (JENSEN, 2003). A causal understanding of CC impacts on biodiversity is critical to determine the vulnerability of the biota to CC (FORTINI; SCHUBERT, 2017; JAESCHKE *et al.*, 2014). Thus, integrative studies that connect patterns, processes, and mechanisms are much needed in ecological climate change impact research (ECCIR; HOFFMANN; SGRÒ, 2011; HOFMANN; TODGHAM, 2010; SOMERO, 2012).

Among all the environmental parameters than compose the Earth's climate, temperature is perhaps the most studied. Environmental temperature has pervasive effects upon all levels of biological organization, from molecular interactions and rates of biochemical reactions, cellular and organismal function, to biogeographical patterns of species distribution (ANGILLETTA, 2009; HOCHACHKA; SOMERO, 2002; SOMERO, 2011). Although CC is altering historical records of both mean temperatures and thermal variation across the globe (BUCKLEY; HUEY, 2016b; EASTERLING, 1997; EASTERLING *et al.*, 2000; IPCC, 2021), ECCIR have focused primarily on biological effects of the former (GARCIA *et al.*, 2014; VÁZQUEZ *et al.*, 2017).

But, even if we simplify climate to its thermal component, mean temperatures clearly are not so biologically relevant in most ecological contexts[1], because organisms rarely experience these conditions. Instead, organisms commonly face temperature fluctuations in their environments (see below), and recent contributions have highlighted the need to pay further attention to the effects of different forms of thermal variability in ECCIR (CLUSELLA-TRULLAS; BLACKBURN; CHOWN, 2011; FOLGUERA *et al.*, 2011; PAAIJMANS *et al.*, 2013; SEDDON *et al.*, 2016; VÁZQUEZ *et al.*, 2017). The predictability of thermal variations in time is another important aspect shaping ecological and evolutionary responses of organisms to their environment (COLWELL, 1974; HOFFMANN, 1978; LEVINS, 1968). Yet, thermal predictability, which will likely decrease with CC (BOTERO *et al.*, 2015; RAFFEL *et al.*, 2013), has been even less investigated in ECCIR (BURGGREN, 2018).

Regarding the study problem, a final comment concerns the importance of *scale* in ECCIR. Thirty years ago, Simon A. Levin referred to scale as "*the fundamental conceptual problem in ecology, if not in all of science*" (LEVIN, 1992). With little empirical evidence on the matter at that time, Levin already recognized that understanding ecological impacts of CC would require integrating multiple spatiotemporal scales, for "*there is no single natural scale at which ecological phenomena should be studied*" (LEVIN, 1992). Since there is not an "all-purpose" scale, proper recognition of the climatic scale(s) that matters for a given biological response is key in ECCIR (BÜTIKOFER *et al.*, 2020; MERTES; JETZ, 2018). Nevertheless, relying on coarse-scaled, widely available climatic data to study ecological impacts of CC has been a common and heavily criticized practice in ECCIR (POTTER; ARTHUR WOODS; PINCEBOURDE, 2013; SEARS *et al.*, 2019; SEARS; RASKIN; ANGILLETTA, 2011). Coarse-scaled descriptions of the climate (hereafter *macroclimate*) are by no means uninformative, but problems arise when they are used to infer biological responses to CC with underpinnings on individual-level processes (e.g., physiology, behavior). Individual organisms experience *microclimates*, understood here as the climate at spatial scales compatible with their body size (BARTHOLOMEW, 1964; PINCEBOURDE; WOODS, 2020; POTTER; ARTHUR WOODS; PINCEBOURDE, 2013). Microclimates, although related to the local macroclimate (i.e., measured at the habitat scale), may be spatially heterogeneous and exhibit distinct temporal variation and predictability (BRAMER *et al.*, 2018; DE FRENNE *et al.*, 2021; WOODS; DILLON; PINCEBOURDE, 2015). Thus, understanding how variability and

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[1] Exceptions would be environments with nearly constant thermal conditions as abyssal zones, marine polar environments, caves, among others.

predictability of climatic parameters vary across scales and, ultimately, affect organisms is critical in ECCIR.

2. *Thermal variation and thermal physiology: the role of upper thermal limits*

Organisms are exposed to different forms of thermal variations in their microclimates, which interactively affect their body temperature (T_b) and physiological performance: 1) *temporal thermal variations*, including both "normal" variations[2] and extreme events[3]; and 2) *spatial thermal variations*, given the spatial distribution of microclimates within habitats (BUCKLEY; HUEY, 2016a, 2016b; DOWD; KING; DENNY, 2015; SEARS *et al.*, 2016a; SEARS; RASKIN; ANGILLETTA, 2011). For ectothermic animals, thermal performance curves (TPCs, Fig. 1) constitute the traditional approach to investigate the effects of "normal" temporal thermal variations on organisms' physiology (DOWD; KING; DENNY, 2015). Typically, TPCs describe a non-linear and asymmetric relationship between T_b and physiological performance, which increases from the Critical Thermal Minimum (CT_{min}) up to an optimum temperture (T_{opt}) that maximizes performance, and then rapidly decreases until the Critical Thermal Maximum (CT_{max}; ANGILLETTA, 2009; ANGILLETTA; NIEWIAROWSKI; NAVAS, 2002; HUEY; STEVENSON, 1979). Thus, critical temperatures set the lower (CT_{min}) and upper (CT_{max}) thermal limits for locomotor performance (COWLES; BOGERT, 1944), and so indicate the range of T_b at which individual activity is possible (i.e., the thermal tolerance range, TR = $CT_{max} - CT_{min}$).

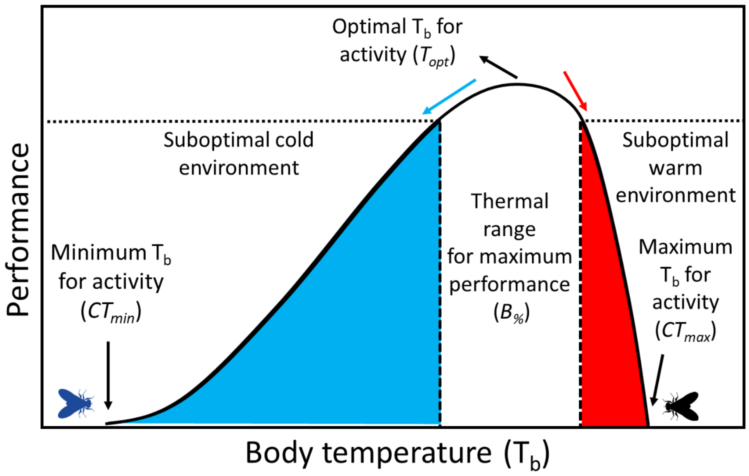

Figure 1. Typical Thermal Performance Curve with its main parameters. Due to Jensen's inequality, performance declines more quickly at suboptimal warm vs. cold environments (warm and blue arrows). Likewise, thermal exposures close to CT_{min} impair individuals but do not kill them (blue fly), but thermal exposures close or a little above CT_{max} may be fatal (black fly). Modified from HUEY & SLATKIN (1976).

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[2] Common fluctuations across generations.

[3] Rare fluctuations that deviate from the population´s history, usually occurring within generations but with potential carry over effects on next generations.

Within TR, thermal fluctuations affect performance differently than a thermal constant regime with the same mean temperature because of Jensen's inequality[4] (DENNY, 2017; DOWD; KING; DENNY, 2015; RUEL; AYRES, 1999; VÁZQUEZ *et al.*, 2017). In other words, for a typical TPC and physiological function, performance decreases more quickly at suboptimal warm vs. suboptimal cold environments (Fig. 1; BOZINOVIC et al., 2011; COLINET et al., 2015; DENNY, 2017; DOWD; KING; DENNY, 2015). Additionally, risks of near-critical thermal fluctuations to individual survival are not symmetric at both sides of a TPC (NAVAS; AGUDELO-CANTERO; LOESCHCKE, 2022), for the chance of abrupt and irreversible damages are higher at the hot than at the cold end (ANGILLETTA, 2009; COLINET *et al.*, 2015; HOCHACHKA; SOMERO, 2002; SOMERO, 2011). Interestingly, wide thermal fluctuations may pose costs on physiological performance while enhance thermal tolerance (particularly CT_{max}) in some species (BOZINOVIC *et al.*, 2011, 2013, 2016; BOZINOVIC; CATALÁN; KALERGIS, 2013; COLINET *et al.*, 2015; FOLGUERA *et al.*, 2011). This apparent trade-off between thermal performance and tolerance could indicate that temperature fluctuations may exert differential selection on different aspects of a TPC. Indeed, extreme and potentially lethal thermal events seem to be the driving factor for the evolution of CT_{max} in many taxa (CLUSELLA-TRULLAS; BLACKBURN; CHOWN, 2011; DUARTE *et al.*, 2012; HOFFMANN, 2010).

In 1967, Daniel H. Janzen published his influential paper on mountain passes, which has been seminal for our understanding of the evolution of thermal limits in response to environmental thermal variations. Janzen's observations of greater thermal variation (annual seasonality) in high latitudes compared to the tropics led him to suggest that species thermal tolerance should match thermal ecology (JANZEN, 1967). In other words, temperate species are expected to have broader TR than tropical counterparts because the former live in more thermally variable environments. Two decades later, George C. Stevens reframed explicitly Janzen's predictions to explain the positive relationship between range size with increasing latitude or elevation (Rapoport's rule; STEVENS, 1989, 1992). According to Stevens, high thermal variability at high latitudes/altitudes would select species with broad TR (*thermal generalists*), which would be able to occupy larger ranges than species at low latitudes/elevations with narrow TR (*thermal specialists*). This relationship between

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[4] A mathematical property of non-linear functions: being $f(x)$ a non-linear function and $\overline{x}$ the mean of a sample of x values, $\overline{f(x)} \neq f(\overline{x})$.

environmental thermal variation and thermal tolerance has been termed the *Climatic Variability Hypothesis* (CVH; GASTON; CHOWN, 1999).

The CVH gained more attention in the context of CC in the last decades, motivating multiple comparative studies of thermal limits across altitudinal or elevational gradients (ADDO-BEDIAKO; CHOWN; GASTON, 2000; COMPTON *et al.*, 2007; DEUTSCH *et al.*, 2008; GUTIÉRREZ-PESQUERA *et al.*, 2016; SUNDAY *et al.*, 2014). Interestingly, most studies have found support for the *CVH* and pointed out to higher interspecific variation in the CT_{min} than in the CT_{max} across latitude/altitude. These observations have led to a notion, termed Brett's rule[5] (GASTON *et al.*, 2009), that CT_{max} evolution is highly constrained among ectothermic animals relative to CT_{min}'s (ARAÚJO *et al.*, 2013; BOZINOVIC *et al.*, 2014; HOFFMANN; CHOWN; CLUSELLA-TRULLAS, 2013). However, it is paramount to recall that Janzen's observations are based on weather station data, and so relate to the macroclimate. Since individual organisms experience microclimates, the evolution of thermal niches should track thermal variations at microclimates faster than at macroclimates (FARALLO *et al.*, 2020). Multiple studies support this idea (CLUSELLA-TRULLAS; BLACKBURN; CHOWN, 2011; DUARTE *et al.*, 2012), and indicate that differences in microclimate promote CT_{max} differentiation among species at the same latitude/elevation (BAUDIER *et al.*, 2018; CHENG *et al.*, 2022; PINTANEL *et al.*, 2019), even within the same community (KASPARI *et al.*, 2015; PINCEBOURDE; CASAS, 2019). The former has three implications: 1) CT_{max} is not as inflexible among ectothermic animals as posed by Brett's rule; 2) the scope of the CVH extends to microclimates (KLINGES; SCHEFFERS, 2021); and 3) considering microclimates is essential to understand aspects of species vulnerability to warming that are based on the upper thermal limits (PINCEBOURDE; CASAS, 2015, 2019; PINCEBOURDE; WOODS, 2020).

3. *A special context: predictability matters.*

Another important consideration relates to the implications of the predictability of thermal variations for thermal limits and its plasticity. Evolutionary theory suggests that natural selection would favor plasticity of thermal limits in thermally variable and predictable environments, promoting anticipatory (e.g., WILLHITE; CUPP, 1982) or reactive responses to extreme temperatures (ANGILLETTA, 2009; HUEY; BENNETT, 1990; PIGLIUCCI, 2001). Conversely, the costs of plasticity in unpredictable environments would constraint its evolution (KINGSOLVER; BUCKLEY, 2018; KINGSOLVER; HUEY, 1998; LANDE, 2014;

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[5] After J.R Brett for his pioneer observations in fish (BRETT, 1956)

SILJESTAM; ÖSTMAN, 2017), so natural selection would favor a strong basal thermal tolerance at the expense of acclimation capacity (ANGILLETTA, 2009; GABRIEL, 2005; GABRIEL *et al.*, 2005; PIGLIUCCI; MURREN; SCHLICHTING, 2006). Thus, the combination of high thermal variation and unpredictability might constitute a scenario of strong selection on thermal limits (ANGILLETTA, 2009; GABRIEL, 2005; GABRIEL et al., 2005; HUEY; KINGSOLVER, 1993; KINGSOLVER; WATT, 1983), particularly for organisms/life stages with limited opportunities to thermoregulate behaviorally[6] (BODENSTEINER *et al.*, 2021; Fig. 2). Few studies, however, have addressed the impact of thermal predictability on thermal limits of ectothermic animals, and the extant evidence is contrasting (BALDANZI *et al.*, 2015; DRAKE; MILLER; TODGHAM, 2017; MANENTI *et al.*, 2014; NIEHAUS *et al.*, 2012; SCHAEFER; RYAN, 2006).

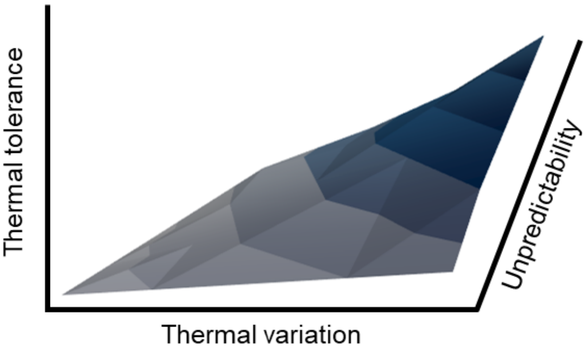

Figure 2. Variability-(un)predictability hypothesis. The joint effects of thermal variations and their degree of unpredictability would favor higher levels of basal thermal tolerance, particularly in life forms with little thermoregulatory opportunities.

4. *Underlying mechanisms*

Despite much research has been conducted on the underlying mechanisms of thermal adaptation (ANGILLETTA, 2009; HOCHACHKA; SOMERO, 2002), our knowledge is largely based on experiments conducted under constant thermal regimes with different mean temperatures (MANENTI; LOESCHCKE; SØRENSEN, 2018). Comparatively, little is known about the molecular machinery necessary to face fluctuating temperatures with varying degrees of predictability. Importantly, evidence suggests that responding to thermal fluctuations may elicit different and independent mechanisms than those involve in the molecular response to constant temperatures (MANENTI; LOESCHCKE; SØRENSEN, 2018; SØRENSEN *et al.*, 2020), as expected from Jensen´s inequality. Thus, the investigation of the molecular mechanisms that respond to thermal variations and thermal predictability, particularly those involving near-critical thermal exposures (SØRENSEN *et al.*, 2020), deserves further attention.

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[6] For instance, sessile life forms like many marine invertebrates or insect pupae, embryos developing within eggs, larval stages in shallow water bodies with little or no thermal stratification, among others.

STANTON-GEDDES et al. (2016) have recently synthesized three mechanistic hypothesis of thermal adaptation that serve an adequate framework to investigate the molecular responses to temporal thermal fluctuations with different degrees of predictability. The *enhanced response hypothesis*, which posits that higher thermal tolerance is achieved through a strong induction of the cellular stress response[7] to repair thermal damages of rare extreme events (FEDER; HOFMANN, 1999; HOFMANN; SOMERO, 1996; SØRENSEN, 2010). Conversely, the *tolerance hypothesis* implies lowering both transcriptional responsiveness and thermal sensitivity of proteins, prioritizing protective[8] over repair pathways, and expressing alternative tolerance mechanisms[9] (BARSHIS *et al.*, 2013; FIELDS, 2001; FRANSSEN *et al.*, 2011; MANENTI; LOESCHCKE; SØRENSEN, 2018; SØRENSEN, 2010; SØRENSEN; KRISTENSEN; LOESCHCKE, 2003). A tolerance strategy might be favored when extreme temperatures are frequent, and so activating constantly the stress response becomes costly (SØRENSEN, 2010; STANTON-GEDDES *et al.*, 2016). A non-mutually exclusive hypothesis proposes *genetic assimilation*, evidenced by a shift from inducible to constitutive expression of stress response mechanisms (MANENTI; LOESCHCKE; SØRENSEN, 2018; SIKKINK *et al.*, 2014; WADDINGTON, 1953). While an enhanced tolerance strategy may typically characterize mechanisms expressed in response to constant thermal regimes (SØRENSEN *et al.*, 2016, 2020; STANTON-GEDDES *et al.*, 2016), predictable thermal fluctuations may require a tolerance strategy under circadian regulation (GRACEY et al., 2008; PODRABSKY; SOMERO, 2004; SØRENSEN; LOESCHCKE, 2002; but see MANENTI; LOESCHCKE; SØRENSEN, 2018). Moreover, if thermal extremes are frequent and unpredictable, a higher basal thermal tolerance may be achieved by a combination of both the tolerance and genetic assimilation strategies, as observed in some lizard and *Drosophila* species from warm environments (MANENTI; LOESCHCKE; SØRENSEN, 2018; SØRENSEN; KRISTENSEN; LOESCHCKE, 2003; ZATSEPINA *et al.*, 2000).

5. *What about spatial thermal variations? The role of behavior in extreme thermal landscapes*

Most of what I have addressed so far, especially in points 3 and 4, concerns primarily to temporal thermal variations. But, as noted before, organisms experience thermal fluctuations in

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[7] E.g., heat shock proteins (HSPs), oxidative stress proteins, protein degradation, apoptosis, among others.

[8] E.g., cytoskeletal organization, metabolic homeostasis, DNA packaging, among others.

[9] E.g., Turandot proteins.

both *time* and *space* (HUEY; BENNETT, 1990; NAVAS *et al.*, 2013; SEARS *et al.*, 2016b; SEARS; RASKIN; ANGILLETTA, 2011). Although it is convenient to separate the temporal and spatial components of thermal variations in experimental settings, studying each dimension in isolation tells us but one aspect of thermal adaptation. In fact, it is not strange that even the most realistic protocols aiming to reproduce temporally fluctuating environments in the laboratory fail to reproduce adaptive patterns found in the wild (KELLERMANN *et al.*, 2015), where temperature varies in time and space. Thus, our understanding of organismal responses to thermal variations requires considering the spatial component of the latter.

Ectothermic organisms integrate both physiology and behavior to respond to the spatiotemporal thermal variations that occur in nature (HUTCHISON; MANES, 1979). Yet, the relative importance of physiological or behavioral responses for organisms in the wild depends on their natural history. Physiological thermal tolerance is expected to be highly important for sessile/developing forms to cope with thermal extremes (see above). On the other hand, behavioral responses will prevail in motile animals that can navigate across spatially heterogeneous thermal environments (hereafter thermal landscapes; SEARS et al., 2016b). Ando so, when encountering extreme temperatures, behavioral avoidance (i.e., thermophobia) is predicted to be the first line of response in motile ectothermic animals (NELSON; HEATH; PROSSER, 1984).

In polybehavioral populations (WOLF *et al.*, 2007), interindividual variation in thermoregulatory behaviors may exist, so that a proportion of individuals may depart from the "average" population response. In other words, alternative behaviors may coexist along with thermophobic responses within a population, diversifying behavioral responses to extreme temperatures despite inherent thermal risks (Fig. 1). Under this rationale, recent studies with small insects have shown that some individuals may actually explore voluntarily extreme hot or cold temperatures, and even engage in thermal risks (NAVAS; AGUDELO-CANTERO; LOESCHCKE, 2022; PORRAS *et al.*, 2021). The latter has been termed thermal boldness by NAVAS et al. (2022) in an investigation with fruit flies (*D. melanogaster*). Interestingly, this newly-described behavior seems to follow predictions of Jensen's inequality, being more evident towards extremely cold vs. extremely hot temperatures when both options were given to fruit flies in an experimental thermal landscape. Moreover, genetically distinct lines of *D. melanogaster* differed in their levels of thermal boldness towards both thermal extremes, opening the question about their potential heritability. Thus, the study of animal behavior in spatially explicit thermal landscapes, including their ecological and evolutionary consequences, is still ripe for investigation.

Objectives and Approach

Main objective

In this Doctoral thesis, we studied physiological and behavioral responses of small ectothermic animals to thermal variations in space and time. We tested the hypothesis that thermal variations at scales relevant to organisms promote physiological and behavioral diversity (i.e., interindividual variation), detectable within populations and among species. We chose two phylogenetically distant group of organisms – anuran larvae and adult fruit flies (*Drosophila melanogaster*) – for conducting this investigation. Given their markedly distinct natural history, the relative importance of physiological and behavioral responses to extreme temperatures should differ between groups. Specifically, physiological thermal tolerance is expected to be highly important for anuran larvae restricted to the aquatic environment (BODENSTEINER *et al.*, 2021; see below), whereas behavior is likely the first response of small and motile fruit flies to thermal extremes in terrestrial environments (NAVAS; AGUDELO-CANTERO; LOESCHCKE, 2022). Accordingly, our specific objectives were:

Specific objectives

1. **Understanding the functional and evolutionary relationships between thermal variations and the upper thermal limits of anuran larvae, including the spatial scale at which they occur.**

Thermal limits for activity (CT_{min} and CT_{max}) are physiological traits that respond to acute thermal exposures (minutes to hours), and so are ecologically relevant to face thermal variations over the course of a day (COWLES; BOGERT, 1944; HUTCHISON, 1961; WILLHITE; CUPP, 1982). In fact, daily thermal variations are key predictors of thermal limits in many lineages of ectothermic animals, including anuran larvae (CLUSELLA-TRULLAS; BLACKBURN; CHOWN, 2011; DUARTE *et al.*, 2012; GUTIÉRREZ-PESQUERA *et al.*, 2016; HOFFMANN, 2010). At this respect, anuran larvae are ideal models to tailor this specific objective for a number of reasons. Even though anuran larvae can thermoregulate behaviorally (HUTCHISON; DUPRÉ, 1992), opportunities to do so may be limited in their aquatic microhabitats, especially in shallow or shaded water bodies with little or no thermal stratification (BALOGOVÁ; GVOŽDÍK, 2015; HADAMOVÁ; GVOŽDÍK, 2011). Moreover, given the thermal properties of water, T_b of small anuran larvae may be isothermal with their surrounding environment (AGUDELO-CANTERO; NAVAS, 2019; NAVAS *et al.*, 2010). For these reasons, selection on CT_{max} is predicted to be stronger on aquatic larvae than in terrestrial adult anurans (BODENSTEINER *et al.*, 2021). Finally, the thermal tolerance of anuran larvae has gained renewed attention in the literature during the last decade (CHENG *et al.*, 2022; DUARTE *et al.*, 2012; GUTIÉRREZ-PESQUERA *et al.*, 2016; KATZENBERGER *et al.*, 2014; PINTANEL *et al.*, 2022), which grants ample room for comparison with results found here. With all this in mind, we designed two independent but complementary studies to address this specific objective:

a) Our starting point was to understand the extent to which microclimates of anuran larvae are *per se* variable in time and across space. Then, in our first study (**Chapter 1, intended journal: *Biotropica***) we characterized the microclimatic thermal diversity among aquatic microhabitats of anurans within a small area (~ 1 km^2) of the Brazilian Atlantic Forest. Previous studies in tropical forests have shown that microclimates of a variety of ectothermic animals, including anurans, may exhibit high spatial thermal heterogeneity at very short distances (ORTEGA CHINCHILLA, 2019; PINCEBOURDE *et al.*, 2016; SCHEFFERS *et al.*, 2017). All these studies focused on terrestrial microhabitats though, so whether the same occurs across aquatic microhabitats remains poorly understood. Regarding factors associated to thermal variations in aquatic microhabitats of anuran larvae, current evidence indicate that canopy cover and physical characteristics of water bodies are relevant (CHENG *et al.*, 2022; DUARTE *et al.*, 2012; GUTIÉRREZ-PESQUERA *et al.*, 2016; PINTANEL *et al.*, 2022; SANABRIA *et al.*, 2021). Specifically, water bodies with little or no canopy tend to be more thermally variable than shaded counterparts, and ponds are generally more variable than streams. Thus, we acquired water temperature data via dataloggers from a variety of water bodies used by anuran larvae of different species within our study area, as well as from a weather station data (air temperature, 2 m above the ground). Temporal extent of data recording encompassed a whole reproductive season plus two short-term samplings (within-generation temporal extents). We characterized and compared microclimatic thermal profiles among water bodies, including thermal predictability, and contrasted them with the thermal profile of the local macroclimate. We predicted that, despite being influenced by the same local macroclimate, aquatic microhabitats of anuran larvae are microclimatically diverse and display high spatial thermal heterogeneity, given differences in their physical structure and associated canopy cover.

b) Understanding whether and how the thermal environment of anuran larvae may vary at a microgeographic scale, we wanted to know whether anuran larvae might correspondingly exhibit physiological diversity in their upper thermal limits (CT_{max}). We tackled this possibility in our second study **(Chapter 2, intended journal: *Global Change Biology*).** Here we compared the CT_{max} among seven anuran species that inhabit the study area targeted in Chapter 1. We collected larvae of these species across distinct water bodies and measured their CT_{max} shortly after in the field at two different experimental heating rates (HRs), a factor known to affect CT_{max} in ectothermic animals (AGUDELO-CANTERO; NAVAS, 2019; CHOWN *et al.*, 2009; MORA; MAYA, 2006; RIBEIRO; CAMACHO; NAVAS, 2012; TERBLANCHE *et al.*, 2007). This approach allowed us to capture immediate physiological responses of tadpoles while accounting for their recent (24-48 h) microclimatic exposure (HOFFMANN; SGRÒ, 2018). We investigated the contribution of historical (phylogenetic relatedness), environmental (microclimate), intrinsic (body mass, ontogeny), and methodological (HRs) factors on interspecific CT_{max} variation. Moreover, we calculated the *Warming Tolerance* (*WT*) index (DEUTSCH *et al.*, 2008), the difference between CT_{max} and T_{max} of the microhabitats (DUARTE *et al.*, 2012), to estimate the relative vulnerability of species to acute warming. However, given that *WT* shall vary in concert with variation on its components (CLUSELLA-TRULLAS *et al.*, 2021), we investigated how factors affecting CT_{max} and the operationalization of T_{max} affect *WT* estimates and diagnosis of vulnerability to acute warming in our study anuran assemblage. In line with the Climate Variability Hypothesis (see above), we predicted that species inhabiting the warmest and more variable water bodies would be the most heat tolerant. Paradoxically, these species were expected to be the most vulnerable to acute warming (lower *WT*) within the study assemblage because they would be living close to their CT_{max}.

2. **Characterizing the behavioral strategies used by fruit flies (*Drosophila melanogaster*) to navigate across extreme thermal landscapes (Chapter 3, intended journal: *Nature Ecology & Evolution*).**

Fruit flies are excellent models to address this study for several reasons. Despite much research has been conducted on the physiological and molecular underpinnings of thermal adaptation in fruit flies (HOFFMANN, 2010; HOFFMANN; SØRENSEN; LOESCHCKE, 2003; SØRENSEN; KRISTENSEN; LOESCHCKE, 2003), less is known about their thermal behaviors (RAJPUROHIT; SCHMIDT, 2016). Fruit flies have impressive dispersal capabilities, being able to travel over 15 km (ca. 6 million body sizes!) through the desert in a single night (COYNE *et al.*, 1982; LEITCH *et al.*, 2021). Moreover, fruit flies have a degree of cognition compatible with decision making in different contexts (GOROSTIZA, 2018; MILLER et al., 2011). Then, and considering the vast thermal landscape available for fruit flies given their small body size (PINCEBOURDE; WOODS, 2020), behavioral decisions are likely important while navigating in their thermal environment. Finally, but not less important, the possibility to use isogenic lines of flies create an ideal system to investigate animal behavior at the population-level and its evolutionary potential (DAVID *et al.*, 2005; GOROSTIZA, 2018; HOFFMANN; PARSONS, 1988; HOULE, 1992). Thus, we took advantage of the newly-introduced module of Thermal Decision Systems (NAVAS; AGUDELO-CANTERO; LOESCHCKE, 2022) to study navigation behaviors of 25 highly inbred isofemale lines of *D. melanogaster* in extremely hot or cold thermal landscapes (HTL or CTL, respectively). We characterized fly behaviors and calculated the proportion of flies in a sample displaying a given behavior in each thermal landscape. We estimated total levels of genetic variation, broad-sense heritabilities, and evolvabilities of navigation behaviors in each thermal landscape. Finally, we explored the context dependency of navigation behaviors of fruit flies by assessing their correlation (phenotypically and genotypically) between thermal landscapes. We anticipated behavioral diversity at the population level, and potential variation between thermal contexts, considering that extreme cold or heat pose different thermal challenges on physiology (Fig. 1) and activate distinct neural pathways in the fly brain (BARBAGALLO; GARRITY, 2015; FRANK *et al.*, 2015; GALLIO *et al.*, 2011).

Chapter 1:

“Within the smallest grid: local microclimatic thermal diversity in the Atlantic Forest from a tadpole’s perspective”

The study area (top left) and the diversity of water bodies it harbors, where a rich anuran assemblage coexist.

LRH and RRH: AGUDELO-CANTERO et al.

Within the smallest grid: local microclimatic thermal diversity in the Atlantic Forest from a tadpole's perspective

Gustavo A. Agudelo-Cantero[*], Fernando R. Gomes, Carlos A. Navas

Department of Physiology, Institute of Biosciences, University of São Paulo, Rua do Matão 101, Travessa 14, 05508-090 São Paulo, Brazil.

[*] Corresponding author: gustavo.a.agudelo@gmail.com

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Abstract

The study of organismal responses to climate change requires gathering climatic information at the scale individual organisms sense the environment (i.e., microclimates), yet using coarsely gridded climate data (>1 km^2) for that purpose has been a common practice. Microclimates may be highly diverse in space and time, and not even the smallest grid of coarse-scales climatic databases captures microclimatic diversity. We investigated the microclimatic diversity available for anuran larvae within a small patch (~1 km^2) of the Brazilian Atlantic Forest. We tested the hypothesis that within-habitat differences in canopy cover and physical characteristics (temporality, water motion) of water bodies promote high microclimatic diversity among aquatic microhabitats of anuran larvae. We used temperature dataloggers to register water temperature in a variety of water bodies during a whole reproductive season, plus two short-term samplings. We characterized the thermal profile of water bodies and contrasted them with the local (air) temperature measured by a weather station inside our study location. Water bodies filtered differently the local thermal environment and differed remarkably in their daily thermal variation and predictability. Open-area water bodies, especially those temporary, amplified local thermal extremes both day and night and were the most thermally variable. On the other hand, canopy buffered water bodies inside the forests from local thermal extremes, making them cooler and less variable. Our results show substantial microclimatic thermal diversity available for tadpoles within a small area of a subtropical forest, and highlights the role of the canopy as modulator of microclimatic diversity.

*Keywords***:** Scale; thermal variation; thermal predictability; amphibians; water body; daytime; nighttime.

Resumo

O estudo das respostas dos organismos às mudanças climáticas requer a coleta de informações climáticas na escala em que os organismos sentem o ambiente (ou seja, microclimas), mas o uso de dados climáticos em quadrículas grossas (>1 km^2) para esse fim tem sido uma prática comum. Os microclimas podem ser altamente diversos no espaço e no tempo, e nem mesmo a menor quadrícula disponível em bases de dados climáticos em escala grossa captura a diversidade microclimática. Neste trabalho investigamos a diversidade microclimática disponível para larvas de anuros em uma pequena área (~ 1 km2) da Mata Atlântica Brasileira. Testamos a hipótese de que as diferenças dentro do habitat na cobertura do dossel e nas características físicas (temporalidade, movimento) de corpos d'água promovem alta diversidade microclimática entre microhabitats aquáticos de larvas de anuros. Utilizamos carregadores de dados de temperatura para registrar a temperatura de uma variedade de corpos d'água durante toda uma estação reprodutiva e mais duas amostragens de curto prazo. Caracterizamos o perfil térmico dos corpos d'água e os contrastamos com a temperatura local (ar) medida por uma estação meteorológica dentro do local do estudo. Os corpos d'água filtraram de maneira diferente o ambiente térmico local e diferiram notavelmente em sua variação e previsibilidade térmica diária. Os corpos d'água de área aberta, especialmente os aqueles temporários, amplificaram os extremos térmicos locais dia e noite, sendo os mais variáveis termicamente. Por outro lado, o dossel amorteceu a variação térmica dos corpos d'água dentro das florestas dos extremos térmicos locais, tornando-os mais frios e menos variáveis. Nossos resultados mostram uma diversidade térmica microclimática substancial que está disponível para girinos em uma pequena área de uma floresta subtropical e destaca o papel do dossel como modulador da diversidade microclimática.

1. INTRODUCTION

In the last decade, Ecological Climate Change Research (ECCR) has debated a central tenet of ecology – the problem of scale (Levin, 1992) – concerning the study of ecological impacts of climate change (Bütikofer et al., 2020). Major criticisms concern the use of climatic data at coarse spatial (> 1 km^2) and temporal (months, years) resolutions to predict biological responses to climate change, for such scales do not capture the abiotic environment actually experienced by individual organisms (Burggren, 2018; Helmuth et al., 2010; Potter et al., 2013; Sears et al., 2011). Although this is a fact, climate change affects biodiversity at all levels of biological organization and across multiple spatiotemporal scales (Pinek et al., 2020; Scheffers et al., 2016). Consequently, Levin's postulate that "there is no single 'correct' scale at which ecological phenomena should be studied" remains as valid as thirty years ago (Levin, 1992), and calls for a more fluent dialog between question, scale, and approach in ECCR (Mertes & Jetz, 2018). For instance, coarse-scale climatic conditions may correlate with limits of species ranges, so analyses at such scales (e.g., species distribution models) are good to inform overall patterns of species occurrence (Lee-Yaw et al., 2021). On the other hand, individual organisms experience microclimates, hereafter understood as climatic variations at spatiotemporal scales compatible with the body size of the focal organisms in a given taxon (Pincebourde et al., 2021; Potter et al., 2013; Woods et al., 2015). Thus, to addressing climate change impacts on individual organisms, and consequences thereof at higher levels of biological organization, studies on microclimates are key (Hannah et al., 2014; Johnston et al., 2019).

That the scale of climate that matters to individual organisms is the microclimate is no novelty, and it has been acknowledged by plant ecologists (Laessle, 1961; Nicolai, 1986) and organismal biologists (Bartholomew, 1964; Cowles & Bogert, 1944; Porter et al., 1973) for decades. Yet, studying microclimates with reference to the local, habitat-level climate (hereafter macroclimate) still bears ecological value. Microclimates are not decoupled from the macroclimate – a change in the macroclimate induces shifts in microclimates – but deviate temporarily from it (Barry & Blanken, 2016; Pincebourde & Woods, 2020). Depending on both abiotic (e.g., topography, canopy cover, water depth) and biotic (e.g., social environment, extended phenotypes) filters, microclimates may either buffer or amplify macroclimatic fluctuations and extremes (Pincebourde & Woods, 2020; Woods et al., 2015, 2021). Understanding the links between the macroclimate and microclimates has been essential to modeling the latter (e.g., Kearney & Porter, 2017; Maclean et al., 2019), considering that most climatic data (hence, our knowledge of climate change) are basically macroclimatic (IPCC, 2021). Furthermore, abiotic and biotic filters create local microclimatic diversity, i.e., spatial heterogeneity in microclimates within habitats (Suggitt et al., 2011; Woods et al., 2015). However, our knowledge about the microclimatic diversity that is locally available to populations remains scant for most organisms and environments (Woods et al., 2015). Filling this gap is essential, for local microclimatic diversity may increase the chance for populations to persist under climate change through behavioral selection of suitable microclimates by motile individuals (Dobkin et al., 1987; Nadeau et al., 2022; Suggitt et al., 2018).

Temperature is a good candidate variable to investigate aspects of local microclimatic diversity for its pervasive impacts on the biology and ecology of living organisms (Angilletta, 2009; Hochachka & Somero, 2002). With few exceptions (e.g., polar seas, abyssal zones, caves), environmental temperature typically fluctuates in both space and time in a scale-dependent manner (Cossins & Bowler, 1987). Therefore, temporal and spatial thermal variation should characterize the diversity of microclimates available to populations at local scales (Woods et al., 2015), especially within spatially complex habitats. This possibility is supported by mounting evidence collected over the last two decades, mostly concerning terrestrial microclimates. For instance, mountain slopes differing in factors like solar radiation and vegetation may exhibit contrasting microclimatic temperatures even over a few hundred meters (Nevo, 1995; Pavlíček et al., 2003). Likewise, forest ecosystems are typically thermally heterogeneous both horizontally and vertically in the understory (Basham & Scheffers, 2020; Klinges & Scheffers, 2021; Scheffers et al., 2017). Canopy cover plays a critical role on this regard by shielding understory microclimates from macroclimatic thermal extremes (Davis et al., 2019; de Frenne et al., 2019), whereas reduced canopy cover amplify macroclimatic thermal fluctuations in microclimates in forest edges and open areas (Ewers & Banks-Leite, 2013; Gols et al., 2021; Worthington-Hill & Gill, 2019). However, aquatic microclimates have received comparatively less attention, particularly in the context of local thermal diversity (Nadeau et al., 2022). It is therefore necessary to take microclimate research to understudied systems if we are to develop a theory to predict local microclimatic diversity (Woods et al., 2015).

Regarding aquatic microclimates, our knowledge about their thermal characteristics has increased over the last decade thanks to an increasing amount of research on the thermal vulnerability of larval anurans (Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016; Oyamaguchi et al., 2018; Pintanel et al., 2022; Sanabria et al., 2021; Simon et al., 2015). In this context, researchers have measured water temperature data on different microhabitats used by larval anurans, shedding light on some typical patterns of microclimatic variation: (a) water bodies inside the forest display lower temporal thermal variation and extreme temperatures relative to counterparts in open areas; (b) temporary water bodies exhibit higher temporal thermal variation and extreme temperatures than those permanent; and (c) lentic water bodies (e.g., ponds) are more thermally variable in time than those lotic (e.g., streams). These trends, however, result mostly from comparing water bodies across macroecological gradients (e.g., latitude, elevation, biomes), which necessarily implies concomitant differences in habitat and macroclimate. But aquatic microclimates of larval anurans may differ considerably over tens of meters or less (Freidenburg & Skelly, 2004; Nadeau et al., 2022), given among-microhabitat variation in e.g., depth, size, water motion, canopy cover, within the same habitat (Geiger et al., 1995; Oke, 1987). Then, aquatic microclimates are likely to be thermally heterogeneous at local scales, i.e., within the same habitat and under the influence of the same macroclimate. Nevertheless, an explicit assessment on this regard is lacking.

The aim of this study is to investigate the microclimatic thermal diversity that is locally available for an anuran assemblage within a small subtropical patch (ca. 1 km^2) of the Atlantic Forest. We chose larval anurans as reference organisms to study aquatic microclimates for several reasons. First, amphibians are a group of high conservation concern (Harfoot et al., 2021; Hoffmann et al., 2010), with many species undergoing massive population declines due to habitat loss, infectious diseases, pollution, invasive species, climate change, among other drivers (Becker et al., 2007; Pounds et al., 2006; Sodhi et al., 2008; Wake & Vredenburg, 2008). Several of these drivers (e.g., habitat loss, climate change) are expected to affect amphibian microclimates (Nowakowski et al., 2017), and in turn microclimates are essential for amphibians to respond to others (e.g., infectious diseases, climate change; Barrile et al., 2021; Scheffers et al., 2013). Then, information on amphibian microclimates is key to assessments of extinction risk and for guiding conservation actions (Hoffmann et al., 2021; Storlie et al., 2014). Second, larval anurans are not isolated in their microhabitats but interact with a number of species within a community. With caution, given caveats posed by measuring method (see *Methods* section), data on aquatic microclimates of larval amphibians may be also informative regarding syntopic interacting species (Katzenberger et al., 2021; Pintanel et al., 2021). Third, and in a broader context, current evidence (discussed above) allows us to test whether patterns of microclimatic variation observed across macroecological clines can also occur at microgeographic scales and mold local microclimatic diversity.

We hypothesize that among-microhabitat differences in canopy cover, temporality, and water motion drive high microclimatic thermal diversity available for larval anurans at a local scale. We employed a multi-scale spatiotemporal approach to test this hypothesis. First, we characterized potential (medium-term) and realized (short-term) *temporal* thermal conditions (average, variation, predictability) of a range of water bodies used by larval anurans within our study area. Then, we contrasted these patterns among water bodies to determine how temporal microclimatic thermal conditions vary across *space*. Furthermore, we analyzed how *microclimatic* (water) temperatures related to the *local* (air) temperature measured by a weather station inside our study area. We distinguished between daytime and nighttime realized microclimatic temperatures because both periods of the day are ecologically distinct and relate to species activity patterns (Gaston, 2019). Accordingly, we use the terms “diurnal” and “nocturnal” to refer to the light and dark phases of the day respectively, whereas “daily” means the 24 h cycle. We anticipate that temporary water bodies exhibit higher temporal thermal variability and extremes than those permanent, and lentic water bodies are more thermally variable in time than those lotic. Moreover, water bodies inside the forest display lower temporal thermal variability relative to counterparts in open areas. Finally, we expect that forest canopy buffer water bodies inside the forest from local temporal thermal variations, turning them less variable and extreme relative to counterparts in open areas.

2. METHODS

2.1. Study area.

The study area is located at the northwest side of the Intervales State Park (ISP; 24º31’03’’ – 24º11’’36’ S and 48º31’22’’ e 48º3’13’’W), a reserve in the São Paulo State, southeastern

Brazil, about 800 – 900 m a.s.l. (Figure 1a, b; (Furlan & Leite, 2008)). The ISP is within the domain of the Atlantic Forest (AF), one of the world's most diverse, yet endangered ecosystems (Brooks et al., 2002; Myers et al., 2000). The once contiguous AF has been severely fragmented, with the most recent estimate of vegetation cover founding only 28% of its original extension (Rezende et al., 2018). Nevertheless, the AF still harbors one of the largest percentages of endemic vertebrate species in the world (Carnaval et al., 2009). For amphibians only, there have been documented 625 species (about 7.4% of global amphibian species richness; Frost, 2021) occurring in remnant fragments of the AF, and 77.6% are endemic (Rossa-Feres et al., 2017). In the ISP, 48 amphibian species were documented in the 1990s occurring within an area of ca. 3 km^2 (Bertoluci, 1998; Bertoluci & Rodrigues, 2002), but a more updated assessment is lacking. Although air temperature and total rainfall vary from year to year in the ISP, the average climate in the region characterizes a cold dry season from May to September and a warm wet season from November to March (Pizo & Oliveira, 2000; Zipparro et al., 2005). Most amphibian species in the ISP breed during this warm wet season in a broad range of natural and anthropogenic water bodies (Bertoluci, 1998; Bertoluci & Rodrigues, 2002).

2.2. Data collection.

2.2.1. Potential, medium-term, microclimatic thermal conditions available for larval anurans at the ISP.

We first characterized thermal conditions in a range of aquatic microhabitats of anurans during a reproductive season in the ISP. We conducted an exploratory field work in November 2014 to identify water bodies used by reproductive adults and anuran larvae. Water bodies differed in size, temporality, water motion and degree of vegetation cover (Table 1), and were distributed within an area of ca. 1 km^2 (Figure 1c, Figure 2). We used

HOBO Pendant dataloggers (Onset Computer Corporation, Bourne, MA) to record temperature of water bodies, as recent studies on the thermal biology of larval anurans have done. The choice of this equipment, which sets the scale of microclimate measurement, was rather pragmatic to keep consistency with the literature (cited in the *Introduction*). Most studies assume, rather than test, that water temperatures measured by HOBOs equal operative temperatures of larval anurans because the high heat capacity and thermal conductivity of water (relative to air) would make larval anurans isothermal with their environment. However, HOBOs differ considerably in material and shape from larval anurans, and do not account for the diversity of body sizes, which interact with rates of temperature change and mold interindividual differences in thermal experience (Agudelo-Cantero & Navas, 2019). Therefore, we consider water temperatures measured by HOBOs as raw thermal conditions of water bodies inhabited by larval anurans that can be compared with current and similar data, and not as operative temperatures.

We deployed HOBOs at the bottom of water bodies for this is their coolest region (Newman, 1989; Woods et al., 2015), which larval anurans could eventually select when daily temperatures are highest at the surface (Duarte et al., 2012). Accordingly, our measurements of water temperature in permanent water bodies (max. depth 1.5 m) are rather conservative regarding maximum temperatures, but not in shallow temporary water bodies (max. depth 15 cm) that lack thermal stratification. HOBOs were set up at 10-min recording interval since 0000 h of day 1 (16 November 2014) and recovered on day 182 (16 May 2015), but we consider data only until 2350 h of day 181, totalizing 26,064 data records per water body. We consider these medium-term datasets as thermal conditions potentially available for anurans because amphibian presence in water bodies was only observed at the time of dataloggers deployment, but was not monitored throughout the whole sampling period. In addition, we acquired data on daily minimum (*MIN*) and maximum (*MAX*) air temperatures registered by the weather station of the ISP (open field, 2 m above the ground; Figure 1c) as a reference of the local air temperature during the sampling period.

2.2.2. Realized, short-term, microclimatic thermal conditions for larval anurans at the ISP.

For our second approach, we conducted two one-week surveys to the ISP during 14-23 November 2017 and 03-10 February 2019, encompassing two different reproductive seasons. We detected and monitored presence of anuran larvae in water bodies and deployed HOBOs as before to register data on raw thermal conditions (given caveats on equipment, see above) realized by anurans throughout both sampling periods. Collectively, we sampled twelve water bodies within our study area, including some previously surveyed during the reproductive season of 2014-2015 (Table 1). For a reference of all the anuran species observed across samplings (14 species, 7 with larval stages), see Table S1. We obtained 1,296 data records in November 2017 and 1,008 in February 2019, per water body. Although of short-term, both records inform about microclimatic thermal variation of consequential relevance for metamorphosing anurans (Lowe et al., 2021), specially for fast developing species. For example, one week may comprise 50% of the developmental time for toad tadpoles (*Rhinella icterica* and *R. ornata*; Table S1) at high average and variable temperatures, which are typical of their developmental microhabitats (M. Simon, 2010).

2.3. Data analysis.

2.3.1. Potential, medium-term, microclimatic thermal conditions.

All data analyses and visualization were performed in the R programming environment (version 4.1.2) (R Core Team, 2022), and the data and code supporting the results of this study will be openly available in a public repository after publication. For our first approach, we compared daily patterns of microclimatic thermal variation among water bodies during our sampling period, particularly minimum (*min*) and maximum (*max*) temperatures, as well as thermal ranges (*range* = *max* - *min*). Then we analyzed how daily temperatures of water bodies were related to the local air temperature at the ISP by estimating two metrics: *temperature offsets* and *thermal coupling* (de Frenne et al., 2021). Temperature offsets estimate the absolute difference between daily microclimatic thermal variables and their local-level counterparts (*min-MIN*, *max-MAX*, and *range-RANGE*). For each temperature offset, we fitted general linear models to contrast the evidence in favor of two competing hypothesis about a differential (model 1) or similar (model 2, null) filtering of the local thermal environment among water bodies. We ranked models according to their second-order Akaike information criterion (*AICc*) (Akaike, 1974; Sugiura, 1978) and selected models with $\Delta AICc < 2.0$ (Burnham et al., 2011; Burnham & Anderson, 2002). On the other hand, the thermal coupling (β) informs how much of the change in the local air temperature is transferred into microclimates, assuming a linear relationship (de Frenne et al., 2021). We estimated β as the regression slope of a linear fit between a given microclimatic temperature variable and its counterpart calculated from the local temperature, considering variation among water bodies (e.g., *max* ~ *MAX* × *water body*). We refer readers to the Supplementary R Script for further details on model fitting and selection.

We employed wavelet transformations to medium-term microclimatic temperature series to analyze whether water bodies differed in the predictability of temporal thermal variation (hereafter thermal predictability). Briefly, wavelet analyses decompose a time series into a three-dimensional space (time, scale/frequency, and power) to determine both the dominant modes of variability and how those modes vary in time, allowing to detect the predictability of fluctuating phenomena at different time scales (Steel & Lange, 2007; Tonkin et al., 2017; Torrence & Compo, 1998). We used the WaveletComp R package (Roesch & Schmidbauer,

2018) to conduct the wavelet transformations and analyze predictability in frequency scales ranging from 6 hr to 60 days, and tested the significance of the wavelet power spectra against 100 simulations of surrogate time series under a white-noise process (i.e., random, uncorrelated values).

2.3.2. Realized, short-term, microclimatic thermal conditions.

For our second approach, we estimated relevant statistics regarding central tendencies, dispersion, and extremes discriminating daytime (0700 – 1950 h) and nighttime (0000 – 0650 and 2000 – 23:50 h) thermal conditions according to average light/dark times in the study zone during sampling periods (Astronomical Applications Department of the U.S. Naval Observatory). For central-tendency statistics we calculated diurnal (d-) and nocturnal (n-) averages (*mean*), medians (*t50*), and modal (*mode*) water temperatures. For describing diurnal and nocturnal thermal extremes, we determined minimum (*min*) and maximum (*max*) water temperatures, as well as the 10th (*t10*) and 90th (*t90*) percentile of the temperature distributions. Concerning diurnal and nocturnal thermal variations, we calculated the thermal range (*range* = *max* - *min*), coefficient of variation (*cv*, %), and *rate* of thermal change (°C/hr). The latter is the ratio between the thermal range and time between thermal extremes (i.e., time from *min* to *max* for daytime, and time from *max* to *min* for nighttime).

We explored the association among daytime (d-) and nighttime (n-) microclimatic variables separately through correlation matrices based on the Spearman-rank correlation coefficient (*rs*). We also included data from the local weather station during sampling periods, specifically minimum (d/n *MIN*) and maximum (d/n *MAX*) temperatures, relative humidity (d/n *RH*%), and absolute daily levels of rainfall (*RAIN*, in mm). We ran Principal Component Analyses (PCAs) to summarize at least 90% of the observed correlation among original variables into few informative and uncorrelated PCs. PCAs were run for daytime and nighttime statistics separately, and then we used selected d/n *PCs* as response variables for subsequent model fitting. Afterwards, we fitted five linear mixed-effects models to test the influence of the temporality (temporary vs. permanent), water motion (lentic vs. lotic) and location of water bodies with respect to the forest (inside vs. open areas, indicating presence or absence of canopy cover) on microclimatic thermal conditions. In addition, we included *water bodies ID* and *Date* of data collection as independent random effects in all models to account for the uniqueness of water bodies and dates that were sampled. We selected the best model(s) explaining the data as before, and estimated the strength of evidence supporting a model relative to others by computing *AICc* weights, as well as the evidence ratio (*ER*) between the top-ranked model against alternative models (Burnham & Anderson, 2002). Finally, we computed the relative importance of predictors (*RI*), i.e., the likelihood of a given predictor variable to be in the best candidate model explaining the data, in cases in which more than one model was selected (Barton, 2018).

3. RESULTS

3.1. Potential, medium-term, microclimatic thermal conditions available for aquatic anurans at the ISP.

Overall, water bodies displayed similar average thermal conditions in the medium term, with daily mean temperatures fluctuating closely around 20°C. Daily patterns of thermal variation, however, were very different among water bodies, which did not mirror simply local thermal fluctuations (Figures S1, S2). Notably, most differences in thermal variation, both among water bodies and in relation to the local thermal environment, concerned daily maximum temperatures (Figure S1). While the absolute daily maximum temperature registered by the weather station was 35°C, the same variable hit values between 36.8 – 61.7°C in bromeliads, 31.4°C in the permanent pond, and barely reached 21.6°C in streams. Microclimate-wise, bromeliads were the most thermally variable in time, followed by the permanent pond. Streams, at the other end, were highly thermally constant throughout our sampling period. Differences among bromeliads are also worth mentioning. For instance, Bromeliad_1, located in an open area, reached daily maximum temperatures ≥ 40°C in 46 days out of 181 (25.4%). On the other hand, Bromeliad_3, which was placed under forest cover, exhibited maximum temperatures ≥ 40°C only 15 days out of 181 (8.3%).

Water bodies also differed in the way they filtered the local thermal environment (Figure 3a). For instance, all bromeliads cooled down as much as the local air temperature (temperature offsets ≈ 0) regardless their associated canopy cover. While those bromeliads under canopy also matched closely the local maximum air temperature, the open-area Bromeliad_1 warmed up to 8°C higher than the local thermal environment. On the other hand, the permanent pond and streams (all under canopy) exhibited minimum temperatures up to 3°C warmer and maximum temperatures up to ≈ 6°C cooler than the local minimum and maximum air temperatures, respectively. Consequently, the daily thermal range of water bodies deviated from the daily local thermal range. In relation to the local thermal environment, water bodies either amplified (Bromeliad_1), buffered (permanent pond and streams), or matched closely (Bromeliad_2 and Bromeliad_3) local daily thermal fluctuations. Differential levels of thermal coupling (microclimatic change transferred from the macroclimate) among water bodies explain these patterns (Figure 3b). While on average 1°C of cooling in the local air temperature led to almost 0.8°C of microclimatic cooling in bromeliads, 0.6°C was transferred into the permanent pond, and less than 0.4°C to streams. Likewise, 1°C of local warming was amplified into almost 1.9°C of microclimatic warming in Bromeliad_1 and 1.4°C in Bromeliad_3, whereas Bromeliad_2 gained 0.9°C. The permanent pond and streams, on the other hand, gained less than 0.6°C and 0.2°C of microclimatic warming from 1°C of local warming, respectively.

All water bodies displayed daily thermal cycles that were significantly predictable throughout the sampling period, but the strength of thermal predictability varied in connection with levels of thermal variation (Figure 4). Bromeliads, the most thermally variable water bodies, displayed the strongest daily thermal predictability, followed by the permanent pond and the thermally constant streams (Figure 4a). Bromeliads also displayed a second peak of thermal predictability at the scale of half a day, but not at time periods longer than a day (Figure 4b). On the contrary, the permanent pond and streams did show traces of weekly and monthly thermal predictability (Figure 4b), yet not consistently over the sampling period (Figure 4a).

3.2. Short-term microclimatic thermal conditions realized by aquatic anurans at the ISP.

Broadly, water bodies differed in their short-term thermal conditions in a similar fashion than that observed at the medium term, even when daytime and nighttime conditions were discriminated (Figure S3-S6). Importantly, the local weather (thermal extremes, relative humidity, rainfall) had little influence on short-term microclimatic temperatures of water bodies at both daytime (Figure S7) and nighttime (Figure S8), so we disregarded local weather variables in further analyses.

During the day, thermal conditions of water bodies were mainly characterized by their average and maximum temperatures (d.PC1, 62.3% of total variance), followed by joint patterns of minimum temperatures and thermal variation (d.PC2, 32.6%; Figure 5a). Water bodies location relative to the forest was the main determinant of diurnal average and maximum microclimatic temperatures (*RI* = 100%), followed by water motion (*RI* = 63%) and temporality (*RI* = 21%) of water bodies (Table 2). On the other hand, diurnal minimum microclimatic temperatures and thermal variation were mainly driven by the temporality of water bodies (*RI* = 100%), with weaker influences of water motion (*RI* = 29%) and location relative to the forest (*RI* = 20%) (Table 2). High diurnal temperatures and thermal variation characterized water bodies in open areas, particularly those lentic and ephemeral like bromeliads and the temporary pond (Figure 6a, b). In the latter, diurnal microclimatic temperatures were always higher than the local diurnal temperature, reaching a temperature offset of up to +9°C in hot days (Figure S3). Streams (lotic) and permanent ponds (lentic) inside the forest had cooler and more constant thermal conditions relative to open area counterparts (Figure 6a, b), and typically buffered local thermal extremes (Figures S3, S5). An extreme example of the influence of forest cover on diurnal maximum temperatures and thermal variation is provided by permanent ponds used by *R. icterica* separated only 4 m linear distance (Figure 2d). In hot days (e.g., 09 February 2019), the pond without forest cover (Perm.pond_(Ri)_1) was up to 10°C warmer than the pond under canopy (Perm.pond_(Ri)_2; Figure S5).

At night, water bodies displayed warmer thermal conditions relative to the local air temperature, especially those of permanent nature (Figure S4, S6). Nocturnal microclimatic temperatures were mainly characterized by average, minimum, and maximum temperatures (n.PC1, 70.3% of total variance), and separately by their thermal range and coefficient of variation (n.PC2, 28.5%; Figure 5b). Nevertheless, both nocturnal average and extreme temperatures (nPC1), as well as nocturnal thermal variation (nPC2) of water bodies were determined by the joint influence of location relative to the forest, temporality, and water motion (Table 2). Permanent water bodies, both ponds and streams to a lesser extent, had higher nocturnal temperatures than temporary water bodies, and such differences were more profound in open areas (Figure 6c). But temporary water bodies were more thermally variable at night than permanent counterparts, specially out of the forest (Figure 6d).

4. DISCUSSION

This study, motivated by "the problem of scale" in Ecological Climate Change Research (ECCR; Bütikofer et al., 2020; Chave, 2013; Levin, 1992), investigated the microclimatic thermal diversity that is locally available for an anuran assemblage within an intentionally small area of the Atlantic Forest ecosystem. We corroborated our hypothesis and documented that water bodies used by anurans for reproduction and larval development within only 1 km^2, although presented similar average conditions, differed remarkably in their temporal thermal variation and predictability. Furthermore, water bodies filtered differently local fluctuations in air temperature. We discuss the ecological scope of our outcomes, implications for target organisms, as well as potential gains from this study to both tropical biology and ECCR.

The thermal profile of a water body depends on its energy balance, which results from exchange mechanisms such as convection, radiation, evaporation, rainfall, wind etc. (Oke, 1987). The relevance of these factors varies between daytime and nighttime and are also influenced by the size and depth of a water body, its temporality, water motion, associated canopy cover, among other aspects (Geiger et al., 1995; Oke, 1987). Our studied water bodies differ in all these physical characteristics, and their interactive effects explain our observations. For instance, the small volume and shallowness of the temporary pond and bromeliads confer them little thermal inertia and a quick energy exchange with the local atmosphere (Geiger et al., 1995; Oke, 1987). This fact, and the higher heat capacity of water compared to air, explain the high temporal thermal variability of temporary water bodies and their tendency to match or even amplify the local air temperature. On the other hand, the continuous mass transfer of lotic water bodies spread energy exchanges and homogenize water temperature, granting higher temporal thermal constancy (Oke, 1987; Ward, 1985). Furthermore, canopy cover shields water bodies against wind and solar radiation during daytime and extreme loss of longwave radiation at night (Geiger et al., 1995; Nunez & Sander, 1982), buffering both diurnal and nocturnal temperatures of water bodies inside the forest from local thermal fluctuations. We acknowledge that temporality, water motion, and vegetation cover are continuous rather than discrete features of water bodies. Yet, since our aim was to show that differences on these factors drive patterns of local microclimatic diversity, our categorical operationalization was sufficient. A mechanistic understanding of such patterns does require to quantify water body characteristics, but such an assessment was beyond the scope of this study.

The microclimatic thermal diversity that we report locally for an anuran assemblage is comparable with observations made across macroecological clines. Within a small subtropical forest patch, water bodies displayed from cool and relatively stable thermal profiles typically anticipated in forest settings, to warm and highly variable thermal conditions observed in counterparts from warmer and dryer habitats (Duarte et al., 2012; Freitas et al., 2016; Gutiérrez-Pesquera et al., 2016; Simon et al., 2015). From this fact, one might infer two possible implications. Proximately, we might expect physiological diversity within the anuran assemblage that inhabits the study zone, given the strong coevolution between microclimatic thermal variation, thermal niche (sensu Gvozdik, 2018), and anuran natural history (Carilo Filho et al., 2021; Duarte et al., 2012; Farallo et al., 2020). Specifically, larvae of species developing in temporary water bodies in open areas are expected to be more heat tolerant than those developing in water bodies inside the forest, especially stream-developing species (Pintanel et al., 2022; Sanabria et al., 2021). Furthermore, highly variable and predictable microclimatic thermal conditions may favor thermal plasticity of heat tolerance even in highly tolerant species (Huey & Bennett, 1990; Pigliucci, 2001), as observed in other amphibian (Simon et al., 2015) and insect lineages (Calosi et al., 2008). Ultimately, our results suggest that local thermal diversity of aquatic microhabitats may characterize forest ecosystems even at more tropical latitudes, as has been observed for terrestrial microhabitats (Scheffers et al., 2017). Seventy-six percent of global amphibian species are known to occur in tropical forests (Pillay et al., 2021), which also exhibit world records for amphibian alpha diversity (Bass et al., 2010). Although not the only factor, local microclimatic thermal diversity (in both land and water) might contribute to sustain high levels of amphibian alpha diversity in tropical forests, where species with distinct microhabitat use, reproductive mode, and thermal niche coexist within small areas (Carilo Filho et al., 2021).

We intentionally delimited our study zone to only ca. 1 km^2 to reflect the smallest grid size available at coarse-scale climatic databases (e.g., Fick & Hijmans, 2017; Karger et al., 2017). From such sources one would obtain a single value for a given climatic variable (e.g., temperature, precipitation) averaging conditions over the entire grid. Although useful in specific contexts, coarse-scale climatic data ignore biophysical processes occurring in boundary layers (Geiger et al., 1995; Oke, 1987; Rosenberg et al., 1983), therefore cannot capture the microclimatic diversity that individual organisms experience inside the grid. Also, the temporal resolution of coarse-scale climatic databases does not reflect the timescales of physiological or behavioral responses to environmental changes (Helmuth et al., 2010; Kearney et al., 2012), or discriminate between daytime and nighttime conditions. Nevertheless, we shall not give prevalence to studies on microclimates over those using larger spatiotemporal scales in ECCR, but call for a more fluent dialog between scale, approach and question. Furthermore, we highlight the value that multi-scale approaches have to disentangle how macroclimatic fluctuations are transferred into microclimates and, ultimately, affect organisms (e.g., Anderson et al., 2022; Robertson et al., 2022; Stark et al., 2022). By relying on a multi-scale approach, we observed that microclimatic temperatures rarely matched the local air temperature (with exception of some bromeliads). Instead, water bodies either buffered or amplified local thermal fluctuations, and the presence of vegetation cover (or lack thereof) had a preponderant role in filtering (or maximizing) local thermal conditions on water bodies. Moreover, discriminating between daytime and nighttime relates microclimates directly with species activity patterns, so we encourage researchers to make this distinction and not only averaging microclimatic data on daily patterns.

In conclusion, we showed high microclimatic thermal heterogeneity among aquatic microhabitats of larval anurans within a subtropical forest patch of only 1 km^2. Such local microclimatic thermal diversity would go unnoticed under any macroclimatic assessment. From a tadpole's perspective, local microclimatic thermal heterogeneity shall drive physiological diversity among syntopic species inhabiting our study zone, as documented for terrestrial (González-del-Pliego et al., 2020; Pintanel et al., 2019) and larval anurans (Pintanel et al., 2022) in other localities. Moreover, canopy-driven microclimatic heterogeneity occurring over microgeographic scales may promote phenotypic differentiation within *Rhinella* populations breeding in thermally distinct water bodies a few meters away (Figure 2d), like occurs in some *Rana* species (Freidenburg & Skelly, 2004; Richter-Boix et al., 2010, 2015). Our study adds evidence to the role of canopy cover in buffering aquatic microclimates in the understory from macroclimatic thermal fluctuations (Scheffers et al., 2013), in a similar fashion than that described for terrestrial microclimates (de Frenne et al., 2019, 2021; Zellweger et al., 2020). Then, preserving and managing vegetation cover may prove an efficient strategy to maintain microrefugia for terrestrial and aquatic species in tropical forests (de Lombaerde et al., 2022; Ilha et al., 2018; Nowakowski et al., 2017). Finally, despite being embedded in an aseasonal macroclimatic thermal environment (Janzen, 1967), tropical forests harbor high microclimatic thermal heterogeneity over microgeographic distances that are consequential for species physiological ecology, diversity patterns, and biogeography (Klinges & Scheffers, 2021). Increasing our understanding on microclimates is therefore critical for the conservation of populations and ecological processes, particularly in the highly diverse and threaten tropical forests.

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CONFLICT OF INTEREST

The corresponding author confirms on behalf of all authors that there have been no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

AUTHOR CONTRIUTIONS

Gustavo A. Agudelo-Cantero: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing, review & editing. **Fernando R. Gomes:** Conceptualization, Supervision, Writing, review & editing. **Carlos A. Navas:** Conceptualization, Funding acquisition, Supervision, Writing, review & editing.

DATA AVAILABILITY STATEMENT

The data and the R script used for the analyses that support the findings of this study will be openly available in a public repository after publication.

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TABLES

Table 1. Sampled water bodies within our study location at the Intervales State Park (ISP), categorized according to the area they are located in relation to the forest (inside the *forest* vs. in an *open* area), their temporality (temporary vs. permanent), and water motion (lentic vs. lotic). “N2014-M2015” means data were collected between November 2014 – May 2015, “N2017” indicates data collection occurred in November 2017, “F2019” refers to data collection in February 2019, and “All” means that data were recorded in all sampling periods.

Code	Water body	Area	Temporality	Water motion	Latitude	Longitude	Sampling period
Bromeliad_1	Bromeliad	Open	Temporary	Lentic	24°16′20.1″S	48°24′18.324″W	N2014-M2015, N2017
Bromeliad_2	Bromeliad	Forest	Temporary	Lentic	24°16′19.164″S	48°24′18.107″W	N2014-M2015, N2017
Bromeliad_3	Bromeliad	Forest	Temporary	Lentic	24°16′17.076″S	48°24′17.315″W	N2014-M2015, N2017
Temp.pond	Pond	Open	Temporary	Lentic	24°15′53.352″S	48°24′40.859″W	N2017
Perm.pond_(Ri)_1	Pond	Open	Permanent	Lentic	24°15′50.616″S	48°24′40.968″W	N2017, F2019

Perm.pond_(Ri)_2	Pond	Forest	Permanent	Lentic	24°15′50.868″S	48°24′41.364″W	N2017, F2019
Perm.pond_(Res.h)	Pond	Forest	Permanent	Lentic	24°16′24.384″S	48°24′55.295″W	All
Perm.pond_(Black.L)	Pond	Forest	Permanent	Lentic	24°16′27.876″S	48°24′25.775″W	F2019
Stream_(Adm)	Stream	Open	Permanent	Lotic	24°15′50.616″S	48°24′45.935″W	N2017, F2019
Stream_(Res.h)	Stream	Forest	Permanent	Lotic	24°16′20.352″S	48°25′3.179″W	All
Stream_(Black.L)	Stream	Forest	Permanent	Lotic	24°16′28.092″S	48°24′27.432″W	N2017
Stream_(Res.r)	Stream	Forest	Permanent	Lotic	24°16′13.44″	48°24′54.504″W	All

Table 2. Selected model(s) to explain variation in diurnal and nocturnal short-term thermal conditions realized by anurans in different water bodies at the ISP. Models are presented according to the influence of predictors on response variables resulting from PCAs (see text). *k* is the number of parameters estimated by the model; *AICc* is the value for the second-order Akaike information criterion; *ΔAICc* is the difference in *AICc* values between the top-ranked model and an alternative candidate model; *AICc weight* is the evidence in favor of a candidate model, among the set of models under comparison, to explain the data; and *ER* is the evidence ratio of the *AICc* weight between the top-ranked model and an alternative candidate model. For more details regarding model structures and selection protocol, we refer readers to our Supplementary R Script.

Model	***k***	***AICc***	***ΔAICc***	***AICc weight***	***ER***
Daytime microclimatic temperatures					
Average and maximum temperatures (d.PC1)					
1. Area and water motion, not temporality	6	481.29		0.37	
2. Area	5	481.58	0.29	0.32	1.15
3. Joint but independent influences of area, temporality, and water motion	7	482.73	1.43	0.18	2.05
Minimum temperatures and thermal variation (d.PC2)					
1. Temporality	5	375.98		0.47	
2. Temporality and water motion, not area	6	377.16	1.18	0.26	1.80

3. Area and temporality, not water motion	6	377.92	1.93	0.18	2.63
Nighttime microclimatic temperatures					
Average temperatures and thermal extremes (n.PC1)					
1. Joint but independent influences of area, temporality, and water motion	7	469.27		0.86	
Thermal variation (n.PC2)					
1. Joint but independent influences of area, temporality, and water motion	7	419.40		0.50	
2. Area and temporality, not water motion	6	419.95	0.55	0.38	1.32

FIGURE LEGENDS

Figure 1. The study area within the Intervales State Park (ISP). (a-b) Location of the ISP in the São Paulo State, southeastern Brazil (QGIS 3.22.0, QGIS Association). The study area, denoted by the small red circle in (b), is in the northwest side of the ISP. (c) Satellite view of our study area (Google Earth Pro 7.3.4.8248, Google LLC ®). Sampled water bodies are inside a perimeter of 4.13 km (yellow line) and an area of ca. 1 km^2. The approximate location of the weather station of the ISP within our study area is illustrated by the orange thermometer. A linear scale reference of 100 m is represented by the red line.

Figure 2. Some sampled water bodies at the ISP. (a) Panoramic view of the study location. (b) Temporary pond in an open area (code: Temp.pond). (c) Permanent pond inside the forest (code: Perm.pond_(Res.h)). (d) Permanent ponds used by *Rhinella icterica* for breeding, only 4 m away from each other (*left*, pond totally exposed to sunlight, code: Perm.pond_(Ri)_1; *right*, fully canopy-covered pond denoted by white arrow, code: Perm.pond_(Ri)_2). (e) Stream in an open area (code: Stream_(Adm)). (f) Stream inside the forest (code: Stream_(Res.r)). (g) Bromeliad covered by canopy (code: Bromeliad_3). (h) Bromeliad in an open area (code: Bromeliad_1).

Figure 3. Relationships between medium-term microclimatic (water bodies) and local (air) temperatures during the reproductive season of anurans in 2014-2015 at the ISP. (a) Temperature offsets (T_{micro} - T_{local}) between daily microclimatic vs. local minimum and maximum temperatures, as well as for the daily thermal range. Positive values inform warmer and negative values cooler temperatures at water bodies relative to the local air temperature. An identity dashed line, where temperature at a given water body is equal to the local air temperature ($T_{micro} = T_{local}$) is provided as reference. (b) Thermal coupling (β, $°\mathrm{C}_{\mathrm{micro}}/1°\mathrm{C}_{\mathrm{local}}$)

between each microclimatic thermal variable and the counterpart at the local thermal environment. At $\beta = 1$ (dashed line), temperatures at water bodies match the local air temperature ($T_{micro} = T_{local}$), leading to a corresponding temperature offset of 0 over time. At $\beta = 0$ (solid line), temperature at water bodies fluctuate independently from the local air temperature. At $0 < \beta < 1$, water bodies buffer microclimatic temperatures from the local thermal environment ($T_{micro} < T_{local}$), whereas at $\beta > 1$ water bodies amplify the local air temperature ($T_{micro} > T_{local}$). In both cases, error bars indicate standard errors.

Figure 4. Thermal predictability of potential, medium-term microclimatic temperatures available for larval anurans during the reproductive season of in 2014-2015 at the ISP. (a) Wavelet power spectrum, where the strength of thermal predictability is indicated by the color distribution (right scale), from low (blue) to high (red). Black solid lines identify periods (i.e., time scales, y-axis) where the relative strength of thermal predictability was high, and the white contour lines detect areas on the plot where thermal predictability was significantly different at $P = 0.05$ from a white-noise process (see text). (b) Average wavelet power plots, where the strength of thermal predictability over a particular period (y-axis) is proportional to the average power of the wavelet transformation (x-axis, notice scale changes among water bodies).

Figure 5. Principal Component Analyses (PCA) for realized, short-term microclimatic thermal conditions experienced by anurans at the ISP during both (a) daytime and (b) nighttime. Individual points represent data for a given water body in a given sampling day (either November 2017 or February 2019). The contribution of original variables to a given PC is proportional to the direction of variable vectors (arrows), and angles between two vectors represent the correlation between a pair of variables. Ellipses contain 68% of

observations for water bodies either inside the forest or in open areas. The percentage of variance explained by each PC is given in parenthesis.

Figure 6. Variation among water bodies on realized microclimatic thermal conditions experienced by larval anurans at the ISP (y-axes), in function of water bodies location regarding the forest, temporality, and water motion. (a-b) represent daytime microclimatic thermal conditions, whereas (c-d) mean nighttime. From PCAs (Figure 5), d.PC1 values relate proportionally to average and maximum diurnal temperatures. Positive d.PC2 values inform high diurnal thermal variation, whereas negative d.PC2 values relate to the magnitude of minimum diurnal temperatures (i.e., lower minimum temperatures at d.PC2 values close to 0). Values of n.PC1 convey average temperatures and thermal extremes (maximum and minimum) at night. Positive n.PC2 values inform higher cooling rates at night, whereas negative n.PC2 values relate to higher thermal variation and broader thermal range at night. For simplicity, abbreviated codes mean: B1, Bromeliad_1; B2, Bromeliad_2; B3, Bromeliad_3; TP, Temp.pond; PP1, Perm.pond_(Ri)_1; PP2, Perm.pond_(Ri)_2; PP3, Perm.pond_(Res.h); PP4, Perm.pond_(Black.L); S1, Stream_(Res.h); S2, Stream_(Adm); S3, Stream_(Black.L); S4, Perm.pond_(Black.L).

FIGURES

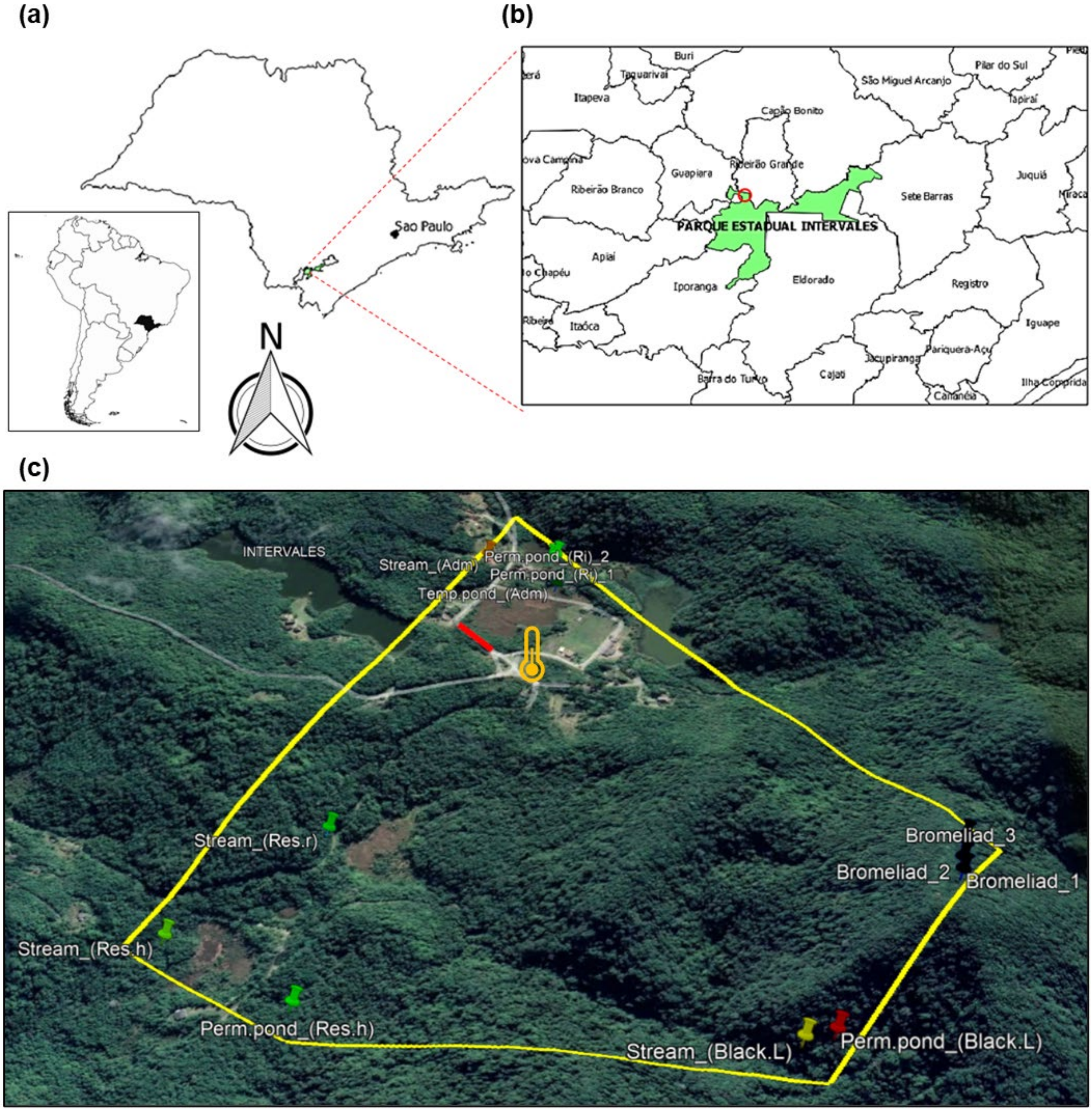


Figure 1

Figure 2

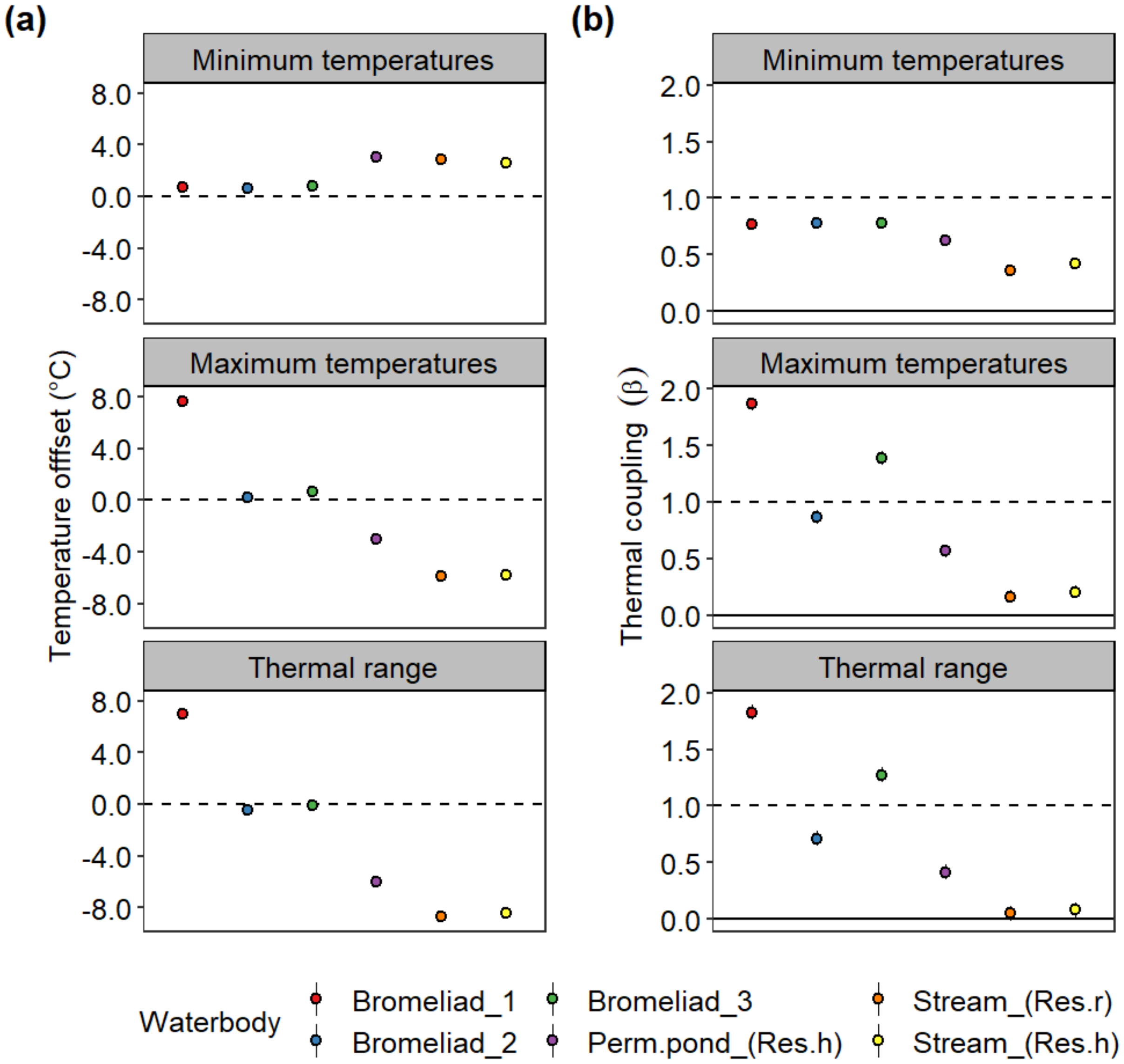

(a)
(b)
Minimum temperatures
Maximum temperatures
Thermal range
Temperature offset (°C)
8.0
4.0
0.0
-4.0
-8.0
Thermal coupling (β)
2.0
1.5
1.0
0.5
0.0
Waterbody
Bromeliad_1
Bromeliad_2
Bromeliad_3
Perm.pond_(Res.h)
Stream_(Res.r)
Stream_(Res.h)

Figure 3

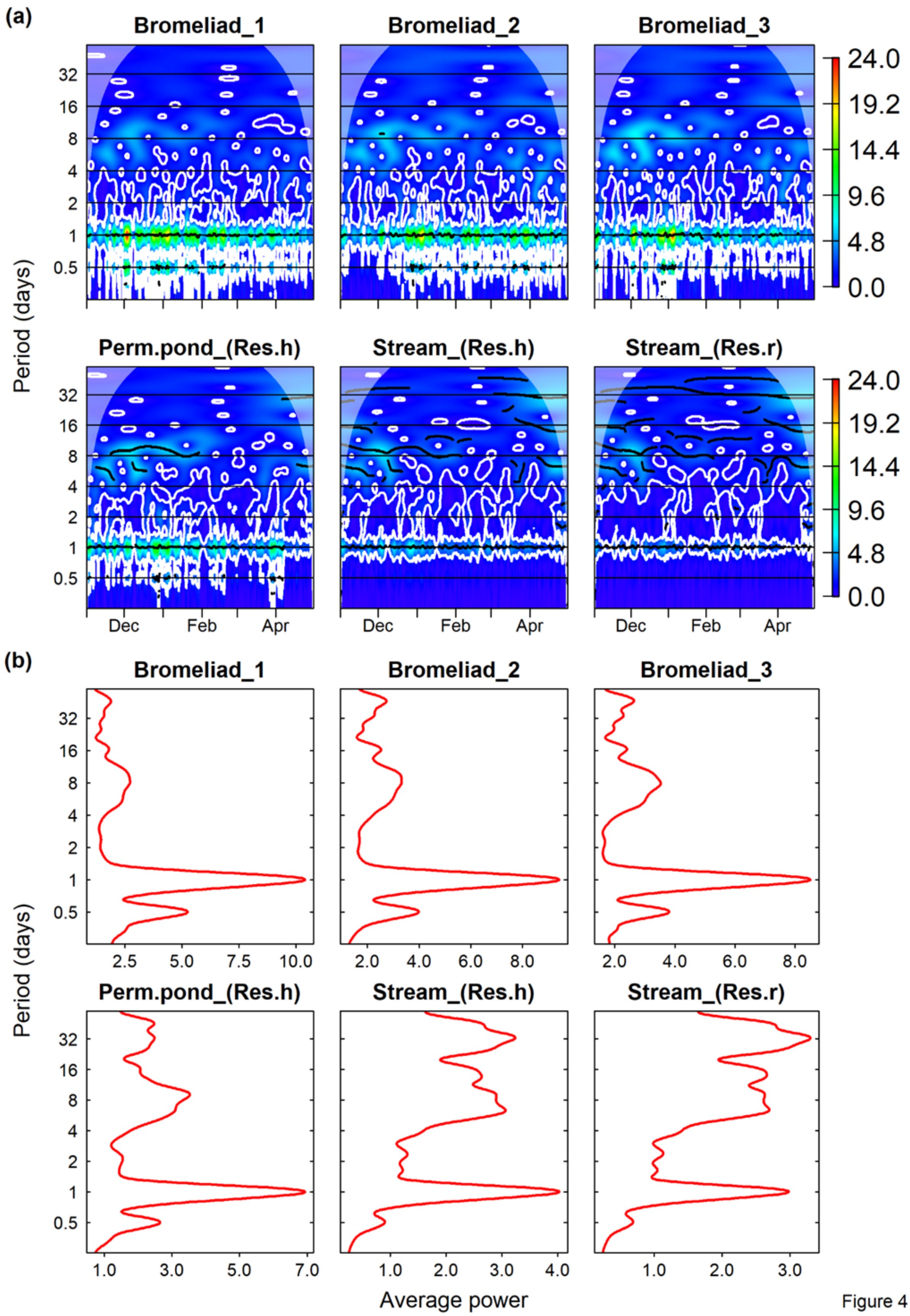

(a)
Bromeliad_1
Bromeliad_2
Bromeliad_3
Perm.pond_(Res.h)
Stream_(Res.h)
Stream_(Res.r)
Period (days)
32
16
8
4
2
1
0.5
24.0
19.2
14.4
9.6
4.8
0.0
Dec
Feb
Apr
(b)
Bromeliad_1
Bromeliad_2
Bromeliad_3
2.5
5.0
7.5
10.0
2.0
4.0
6.0
8.0
Perm.pond_(Res.h)
Stream_(Res.h)
Stream_(Res.r)
1.0
3.0
5.0
7.0
1.0
2.0
3.0
4.0
Average power

Figure 4

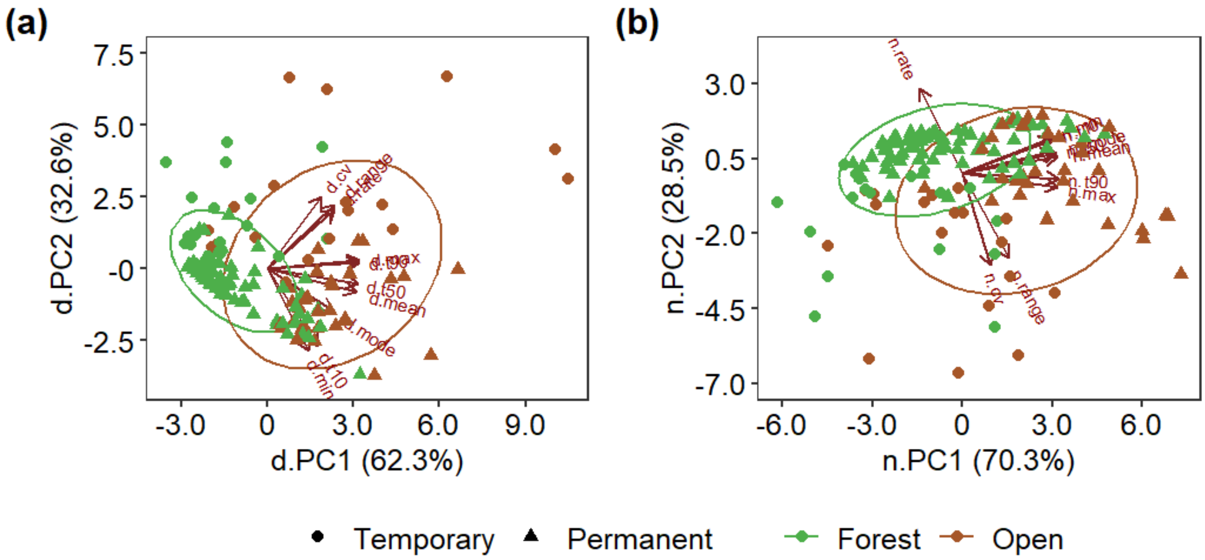

(a)
(b)
7.5
5.0
2.5
-0
-2.5
d.PC2 (32.6%)
-3.0
0
3.0
6.0
9.0
d.PC1 (62.3%)
d.cv
d.range
d.rate
d.max
d.t90
d.t50
d.mean
d.mode
d.t10
d.min
3.0
0.5
-2.0
-4.5
-7.0
n.PC2 (28.5%)
-6.0
-3.0
0
3.0
6.0
n.PC1 (70.3%)
n.rate
n.min
n.mode
n.mean
n.t90
n.max
n.cv
n.range
Temporary
Permanent
Forest
Open

Figure 5

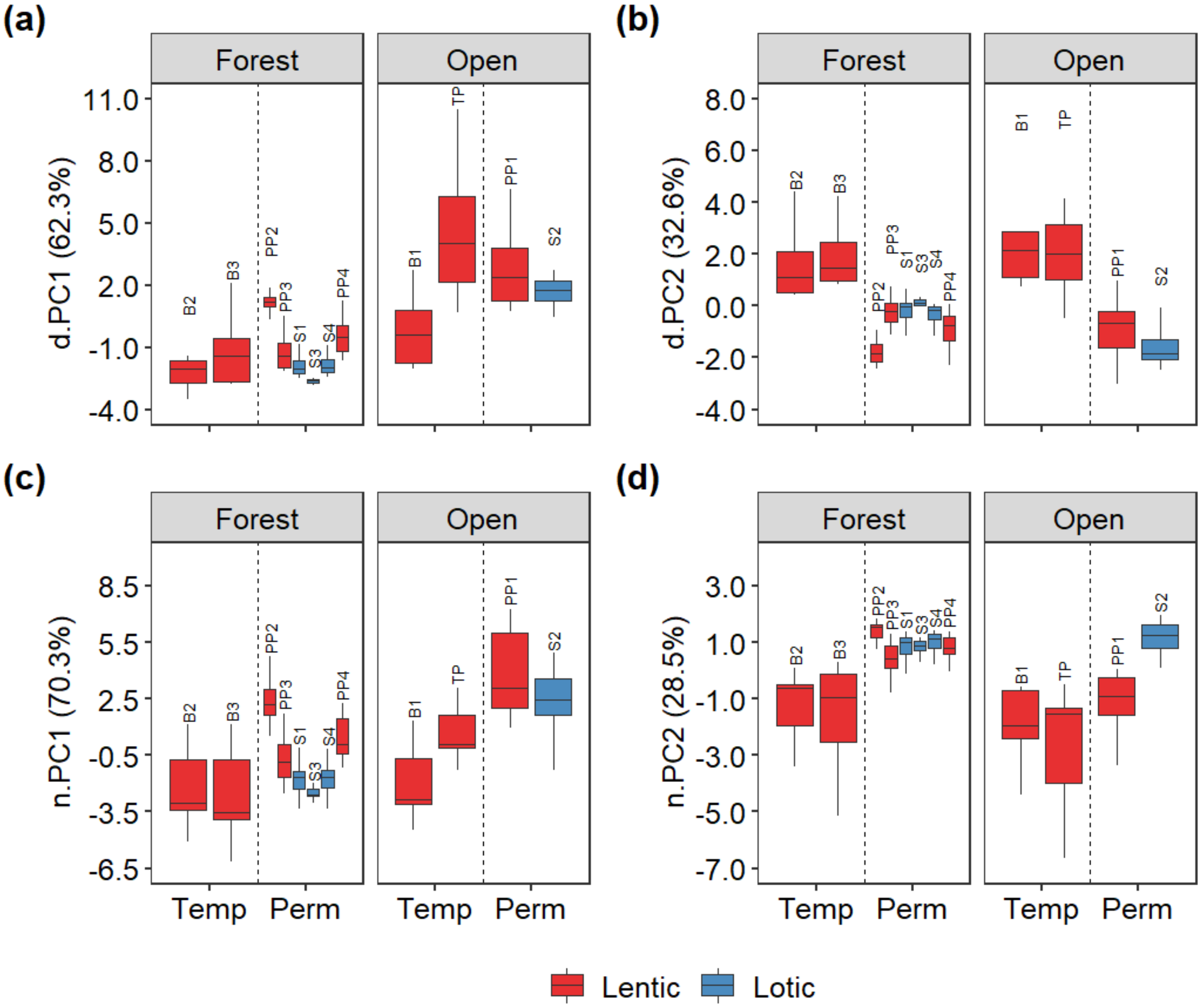

(a)
Forest
Open
d.PC1 (62.3%)
11.0
8.0
5.0
2.0
-1.0
-4.0
B2
B3
PP2
PP3
S1
S3
S4
PP4
B1
TP
PP1
S2
(b)
Forest
Open
d.PC2 (32.6%)
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
B2
B3
PP2
PP3
S1
S3
S4
PP4
B1
TP
PP1
S2
(c)
Forest
Open
n.PC1 (70.3%)
8.5
5.5
2.5
-0.5
-3.5
-6.5
B2
B3
PP2
PP3
S1
S3
S4
PP4
B1
TP
PP1
S2
Temp
Perm
Temp
Perm
(d)
Forest
Open
n.PC2 (28.5%)
3.0
1.0
-1.0
-3.0
-5.0
-7.0
B2
B3
PP2
PP3
S1
S3
S4
PP4
B1
TP
PP1
S2
Temp
Perm
Temp
Perm
Lentic
Lotic

Figure 6

SUPPLEMENTARY INFORMATION

Gustavo A. Agudelo-Cantero, Fernando R. Gomes, Carlos A. Navas

Table S1. Anuran species (grouped by family) observed in the studied water bodies at the Intervales State Park (ISP). Names in bold correspond to species for which larval stages were specifically observed during samplings in November 2017 and February 2019.

	WATER BODY						
SPECIES	**Bromeliad_2**	**Temp. pond**	**Perm.pond_ (Ri)_1**	**Perm.pond_ (Ri)_2**	**Perm.pond_ (Res.h)**	**Perm.pond_ (Black.L)**	**Stream_(Res.h)**
BUFONIDAE							
Rhinella icterica		X	X	X			
Rhinella ornata						X	
HYLIDAE							
Boana faber					X	X	
Boana bischoffi		X				X	
Dendropsophus minutus		X					
Dendropsophus giesleri					X		
Scinax rizibilis					X		
Scinax perpusillus	X						
Trachycephalus mesophaeus					X		
HYLODIDAE							
Crossodactylus caramaschii							X
LEPTODACTYLIDAE							
Leptodactylus latrans		X					
Physalaemus cuvieri		X					
ODONTOPHRYNIDAE							
Proceratophrys boiei						X	
PHYLLOMEDUSIDAE							
Phyllomedusa distincta					X	X	

Figure S1. Time series for daily minimum (blue), mean (black), and maximum (red) microclimatic (water bodies) and local (air, as measured by the weather station) temperatures at the ISP from 16 November 2014 to 15 May 2015.

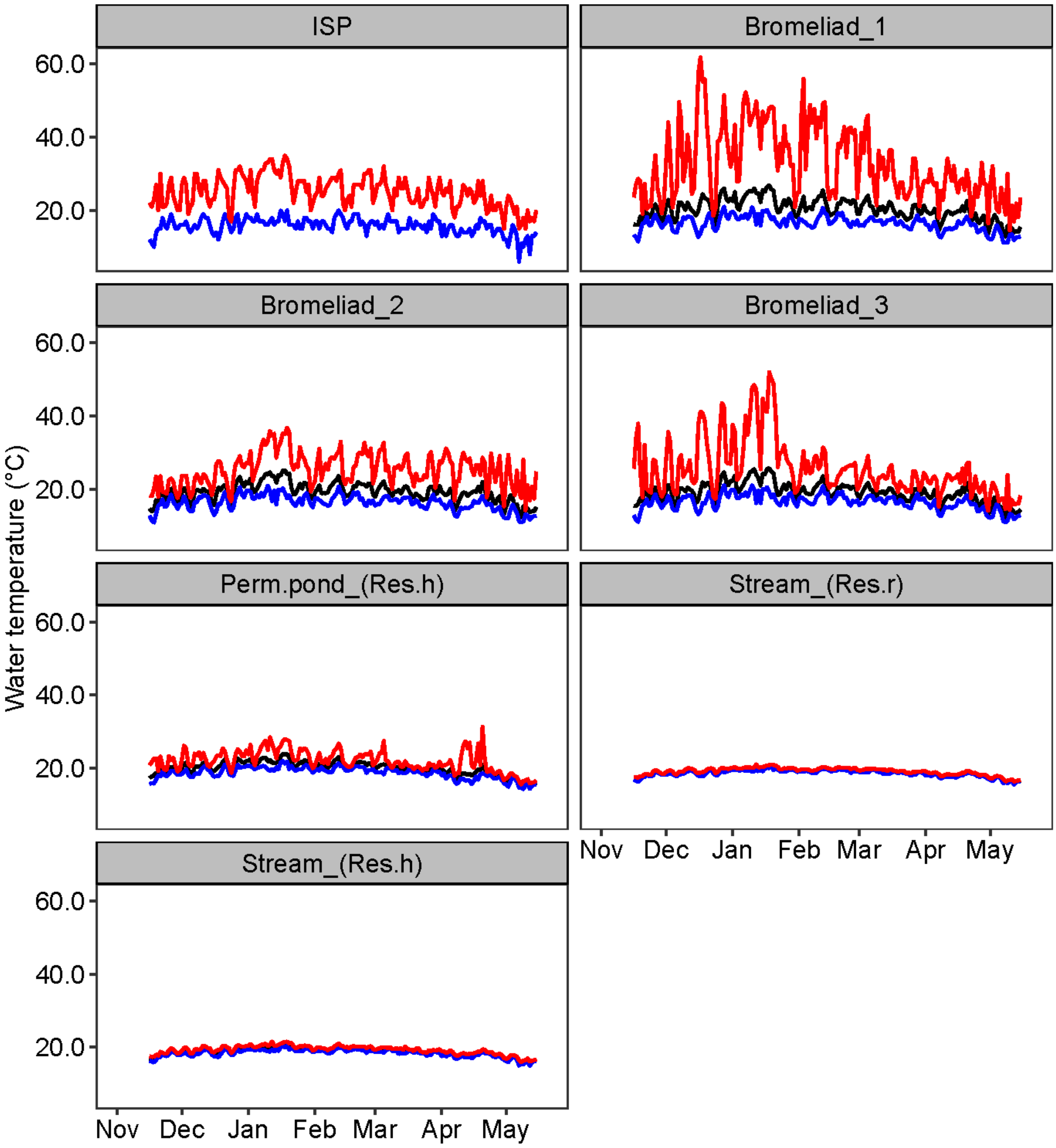


Figure S1

Figure S2. Daily thermal range (difference between daily maximum and minimum temperatures) of microclimates and local thermal environment at the ISP.

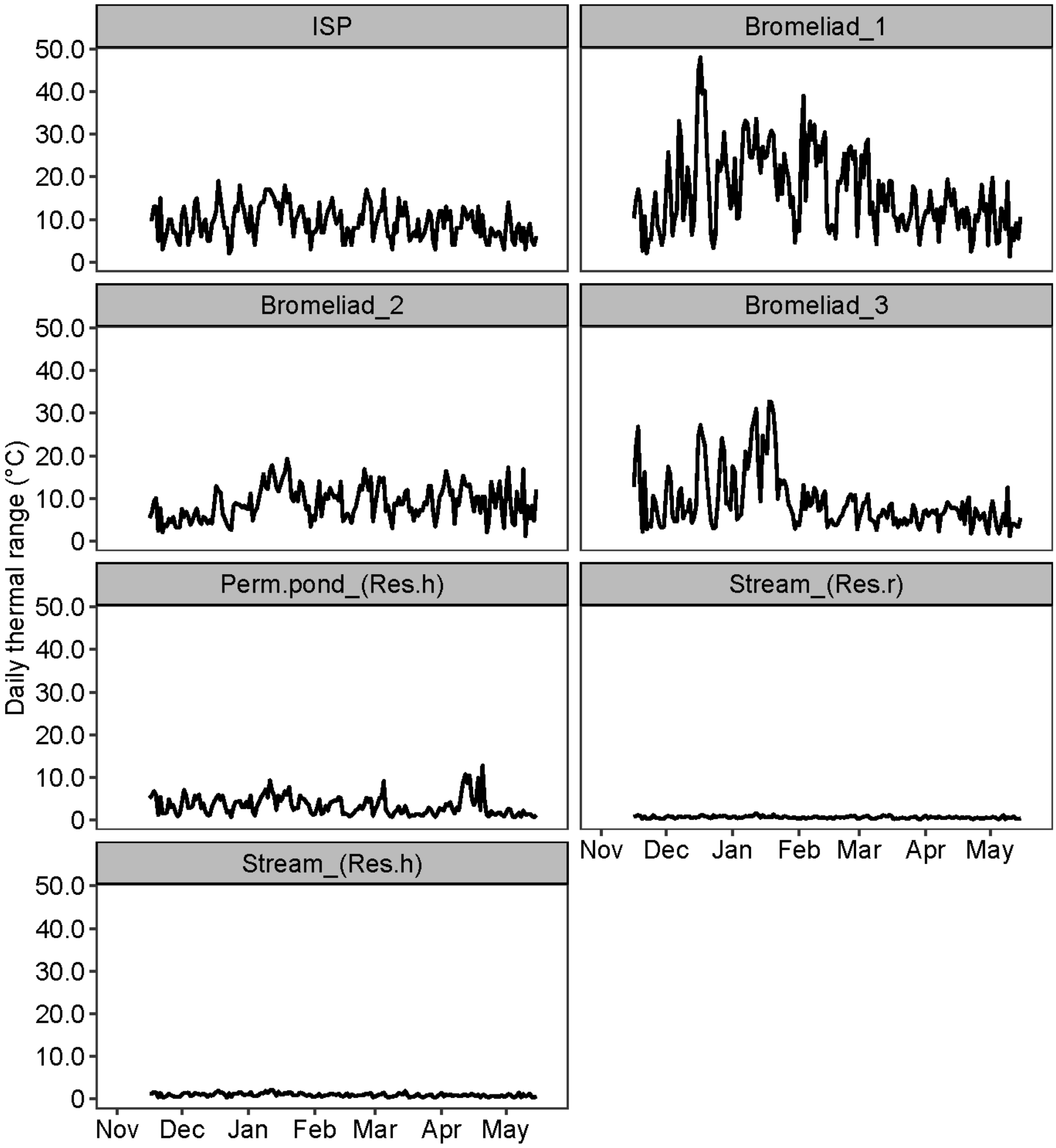


Figure S2

Figure S3. Overall diurnal microclimatic and local thermal conditions between 14-22 November 2017 at the ISP. For water bodies (dashed lines) are displayed the diurnal minimum (d.min), 10th percentile (d.t10), average (d.mean), 90th percentile (d.t90), and maximum (d.max) temperatures. For the local thermal environment (solid lines) are displayed the diurnal minimum (d.MIN) and maximum (d.MAX) temperatures registered by the weather station of the ISP.

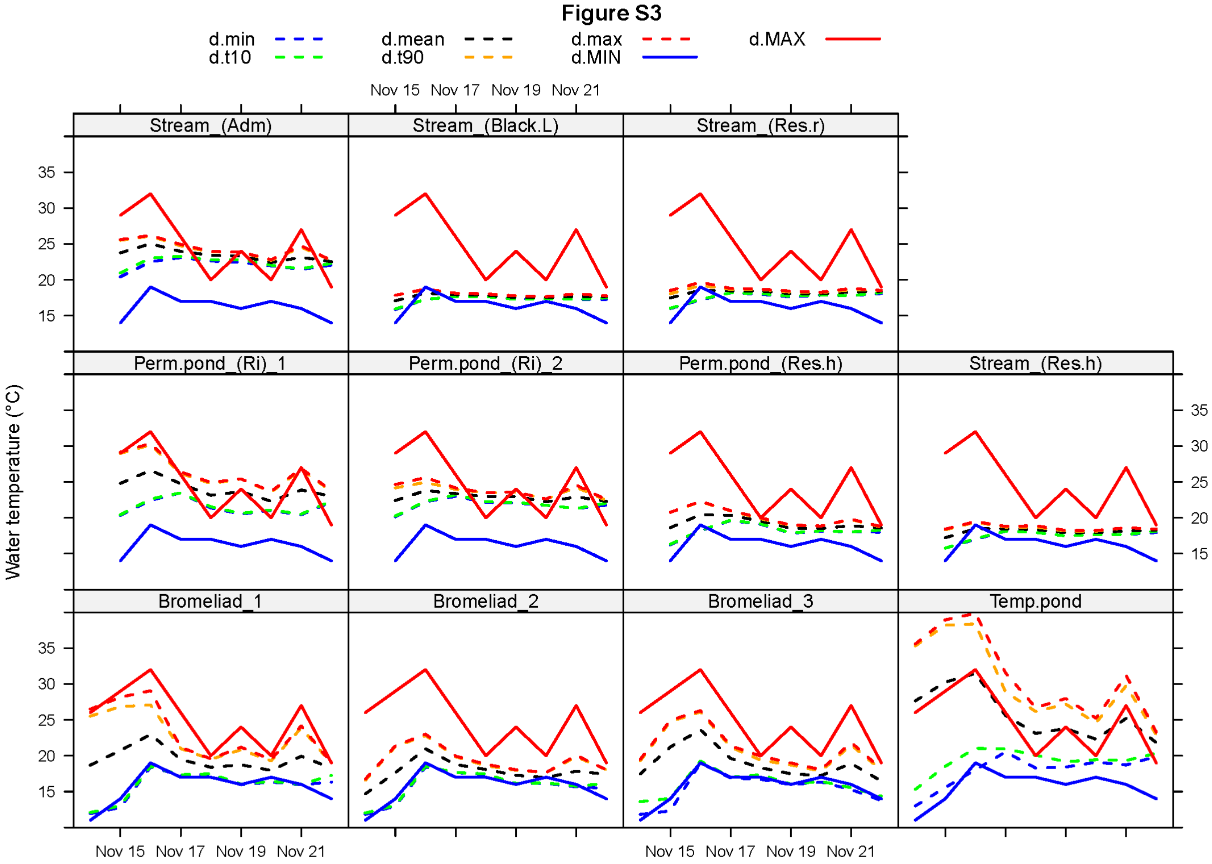


Figure S4. Overall nocturnal microclimatic and local thermal conditions between 14-22 November 2017 at the ISP. For water bodies (dashed lines) are displayed the nocturnal minimum (n.min), 10th percentile (n.t10), average (n.mean), 90th percentile (n.t90), and maximum (n.max) temperatures. For the local thermal environment (solid lines) are displayed the nocturnal minimum (n.MIN) and maximum (n.MAX) temperatures registered by the weather station of the ISP.

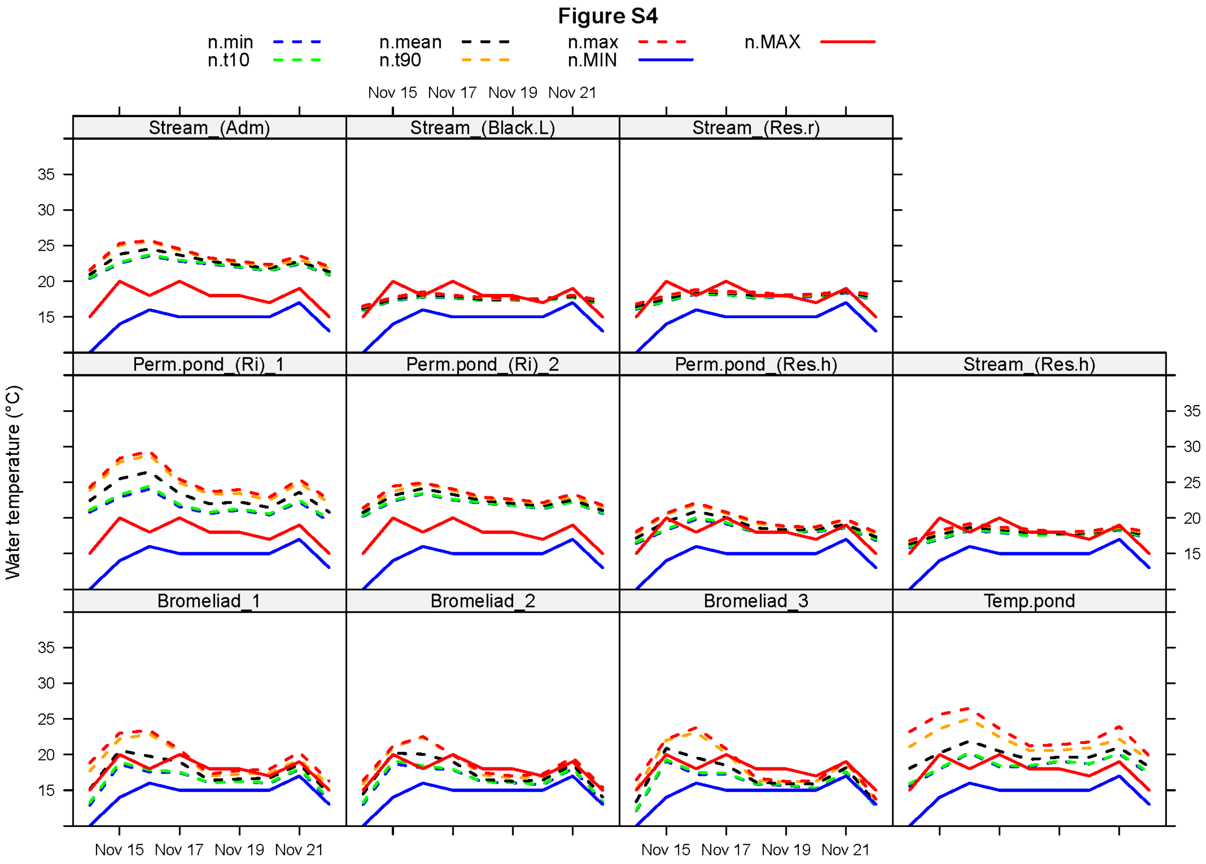


Figure S5. Overall diurnal microclimatic and local thermal conditions between 03-09 February 2019. Figure details as in Figure S3.

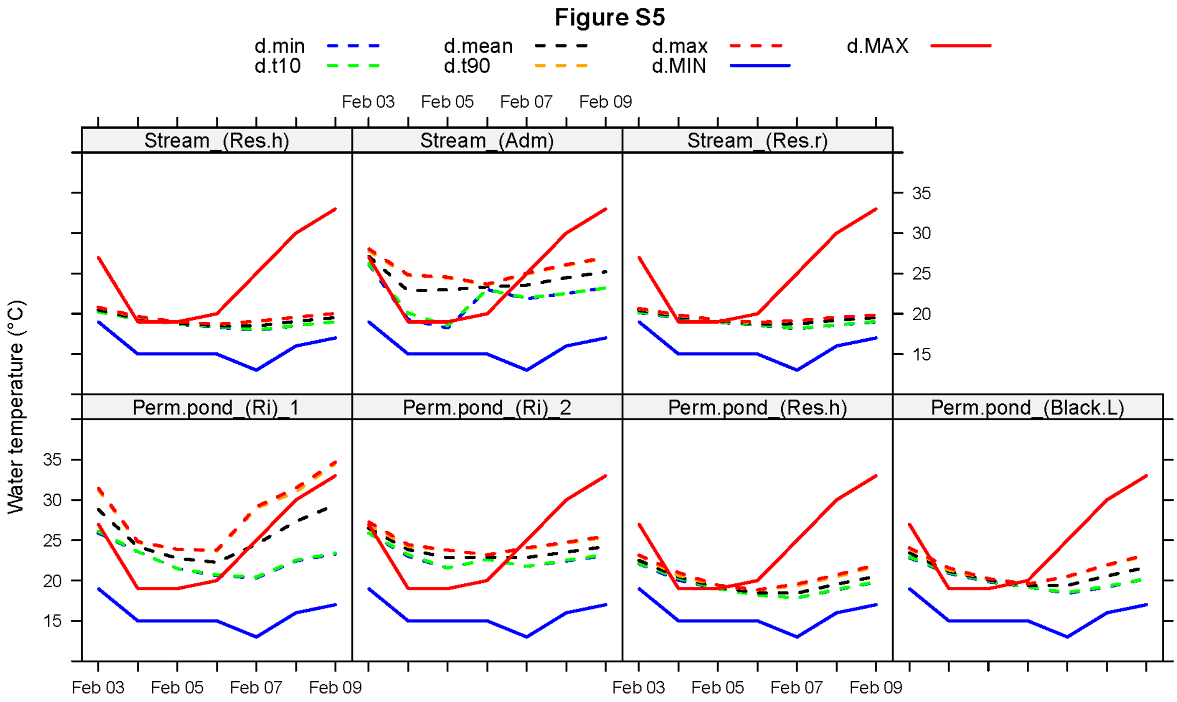


Figure S6. Overall nocturnal microclimatic and local thermal conditions between 03-09 February 2019. Figure details as in Figure S4.

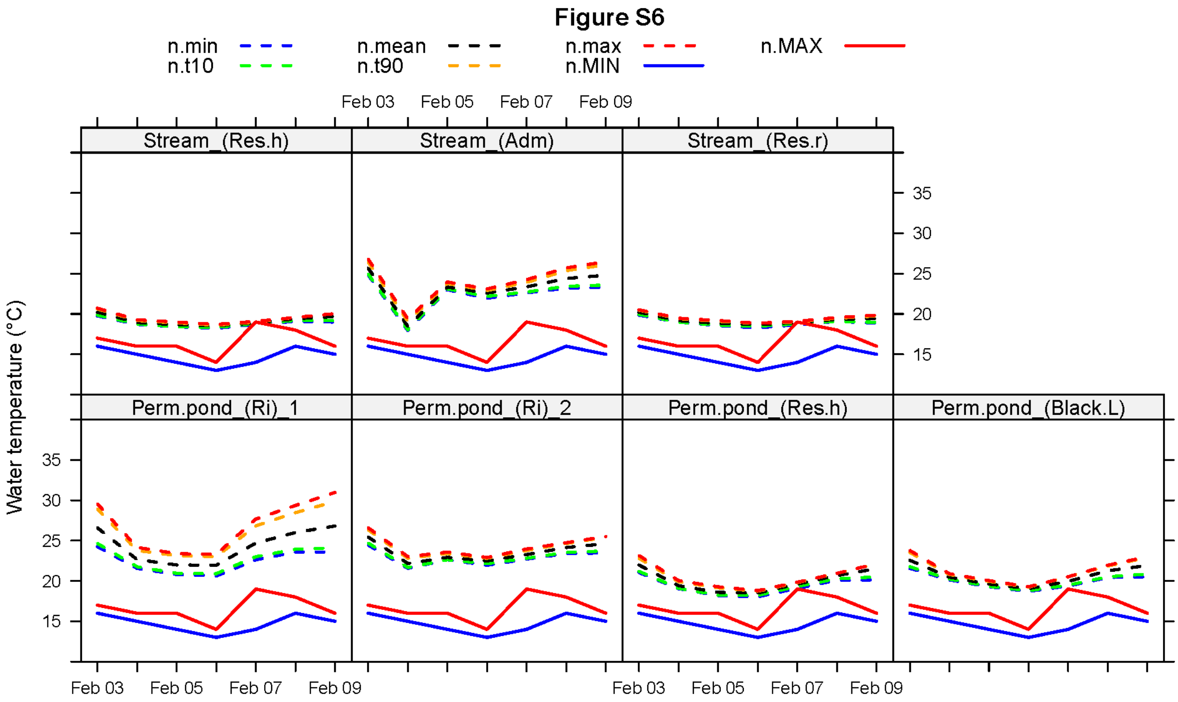


Figure S7. Correlation matrix among variables for diurnal microclimatic (lowercase names) and local (uppercase names) thermal conditions. Besides diurnal microclimatic variables displayed in Figure S3, we included median (d.t50) and modal (d.mode) temperatures, thermal range (d.range = d.max – d.min), coefficient of variation (d.cv, %), rate of diurnal thermal change (d.rate, °C/hr). From the local environment we also included the diurnal relative humidity (d.RH) and absolute level of daily rainfall (RAIN). Blank spaces mean non-significant *rs* values ($P > 0.05$). Blue tones indicate positive significant correlations, red tones indicate negative significant correlations, and size and color intensity of dots inform the strength of the correlations according to *rs* values (right scale). Black squares identify variable clusters with similar patterns of correlation.

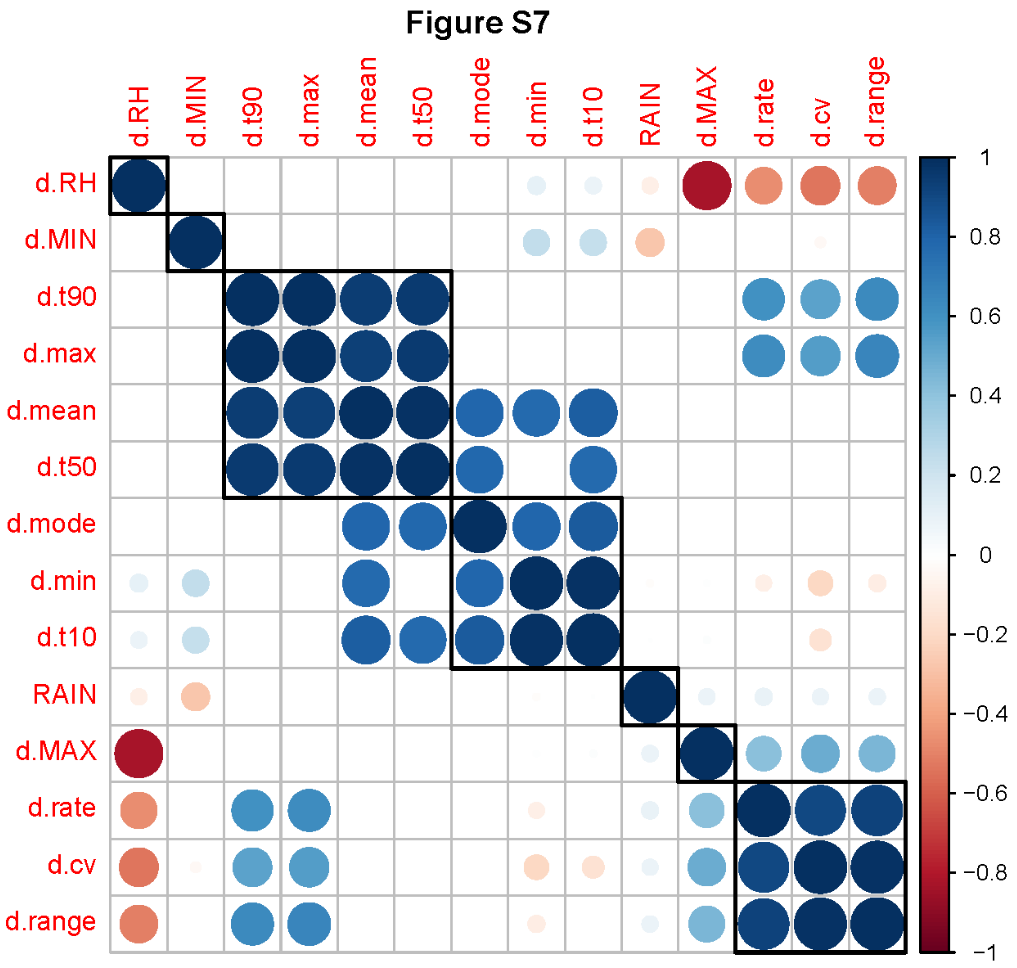
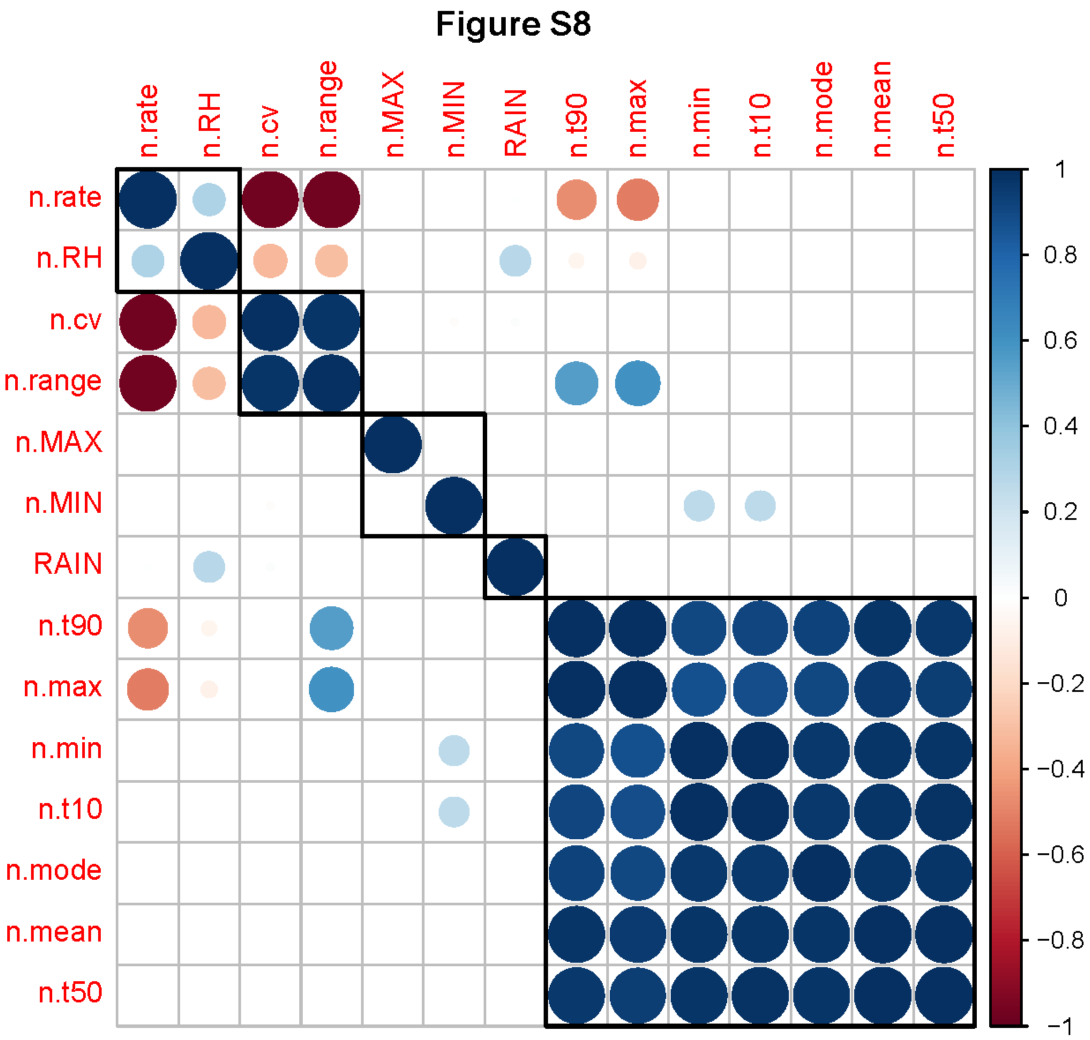
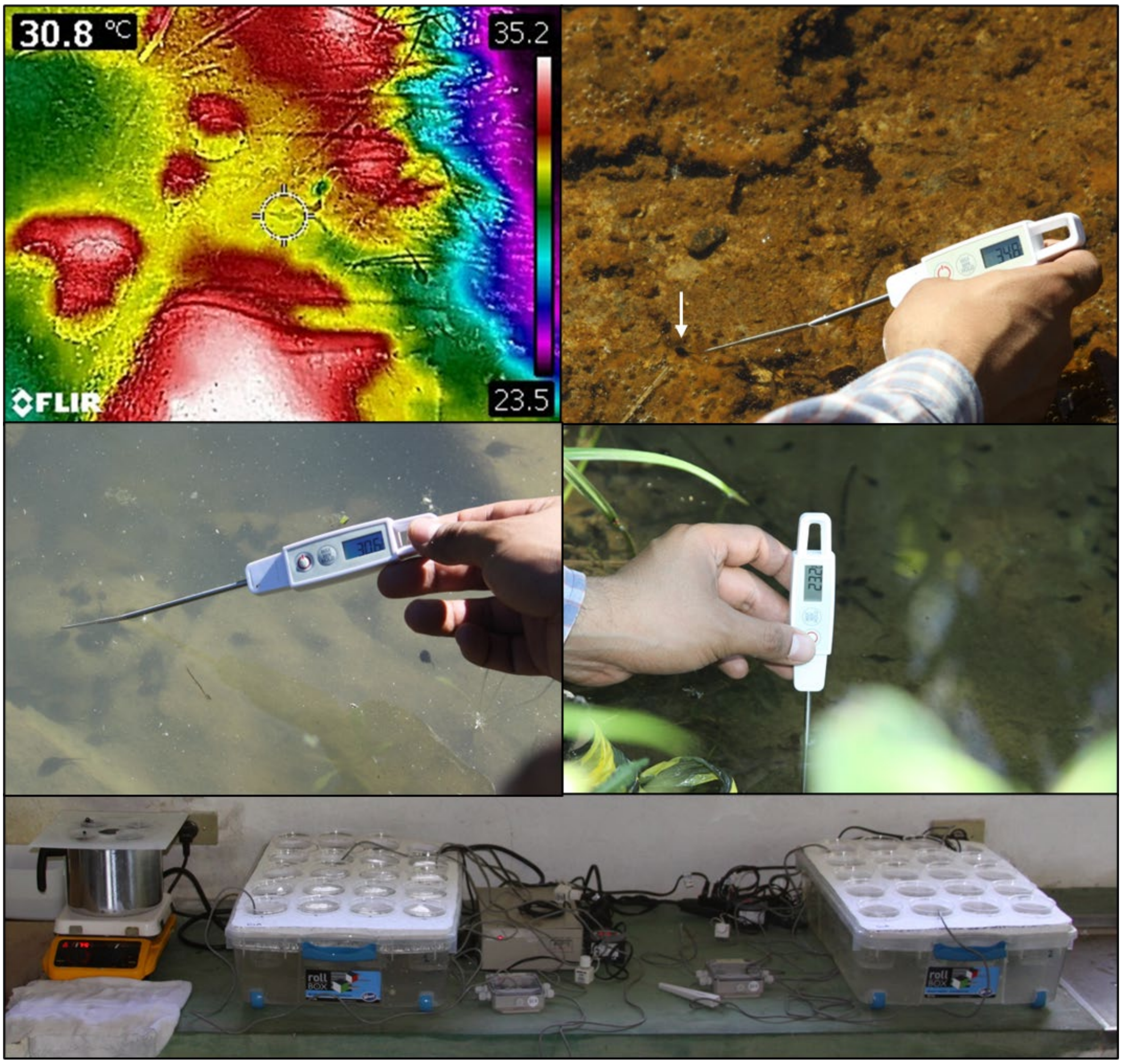


Figure S8. Correlation matrix among variables for nocturnal microclimatic and local thermal conditions. Figure details and variable names ("n." for nocturnal counterparts) as in Figure S7.



Chapter 2:

“Sources of variation at both components of the Warming Tolerance index (WT) affect estimates and inference of vulnerability to acute warming: Lessons from a local-scale study.”



Water bodies used by tadpoles inside our study area differ in their thermal regimes. (Bottom) Equipment used to measure heat tolerance of tadpoles in the field.

Gustavo A. Agudelo-Cantero (ORCID iD: 0000-0002-6649-5523)

Sources of variation at both components of the Warming Tolerance index (*WT*) affect estimates and inference of vulnerability to acute warming: Lessons from a local-scale study.

Running title: Biases of the Warming Tolerance index

Gustavo A. Agudelo-Cantero[1*], Pedro L. Atencia-Gándara[2], Fernando R. Gomes[1], Carlos A. Navas[1].

[1] Department of Physiology, Institute of Biosciences, University of São Paulo. Rua do Matão 101, Travessa 14, 05508-090 São Paulo, SP. Brazil.

[2] Department of Biology, FFCLRP, University of São Paulo. Avenida Bandeirantes 3900, 14040-900 Ribeirão Preto, SP. Brazil.

[*] Corresponding author: gustavo.a.agudelo@gmail.com. +47 929 76 586.

ORCID iDs:
Gustavo A. Agudelo-Cantero (0000-0002-6649-5523)
Pedro L. Atencia-Gándara (0000-0002-9645-9275)
Fernando R. Gomes (0000-0003-2244-6161)
Carlos A. Navas (0000-0002-9859-0568)

Abstract

Identifying the most vulnerable species to climate warming is a top priority of global change biology. The Warming Tolerance (*WT*) index, the difference between the Critical Thermal Maximum (CT_{max}) and a measure of the habitat temperature (e.g., T_{max}), is a popular method used for this purpose, but key aspects of its conceptualization remain ignored. Here we investigated the physiological diversity (CT_{max}) existing in an anuran assemblage that occur over a very small and micro-climatically diverse area of the Brazilian Atlantic Forest. We measured the CT_{max} of freshly-collected tadpoles in the field and examined the contribution of different factors (phylogenetic relatedness, microclimate, body mass, ontogeny, and experimental heating rates - HRs) to interspecific CT_{max} variation. We then assessed the impact of sources of CT_{max} variation and different summary statistics (STs) for T_{max} on *WT* estimates and inference of vulnerability to acute warming. We observed a vast physiological diversity within the assemblage at the smallest spatial scale reported for anurans (~1 km^2). Such local interspecific CT_{max} variation was mostly related to species' microclimates and microhabitat use than to phylogenetic relatedness or individual body mass. Ontogeny had minor effects on the CT_{max} of a target species. Experimental heating rates had different and independent effects on averages and interindividual variance of CT_{max} across species. Both CT_{max}- and T_{max}-related variation affected *WT* in species-wise and microhabitat-wise manners, respectively, but effect sizes of the latter were comparatively higher. Particularly, using a microclimatic ST far from extreme T_{max} led to comparable *WT* values than a macroclimatic ST, and both led to dubious diagnosis of vulnerability to acute warming. Our study demonstrate that proper conceptualization is crucial in *WT*-based vulnerability assessments, and emphasize that the individual-level nature of *WT* sets boundaries to inference of vulnerability to acute warming at higher levels of biological organization.

Resumo

Identificar as espécies mais vulneráveis ao aquecimento é uma das principais prioridades da biologia das mudanças globais. O índice de tolerância ao aquecimento (*WT*), a diferença entre a Temperatura Crítica Máxima (CT_{max}) e uma medida da temperatura do habitat (p.ex., T_{max}), é um método popular usado para esse fim, mas aspectos chave de sua conceitualização permanecem ignorados. Aqui, investigamos a diversidade fisiológica (CT_{max}) existente em uma assembléia de anuros que ocorre em uma área muito pequena e micro-climaticamente diversa da Mata Atlântica Brasileira. Medimos o CT_{max} de girinos recém coletados no campo e examinamos a contribuição de diferentes fatores (filogenia, microclima, massa corporal, ontogenia e taxas de aquecimento experimental - HRs) na variação interespecífica do CT_{max}. Também avaliamos o impacto dessas fontes de variação do CT_{max} e de diferentes estatísticas de resumo (STs) para T_{max} nas estimativas de *WT* e inferência de vulnerabilidade ao aquecimento agudo. Observamos uma vasta diversidade fisiológica dentro da assembléia na menor escala espacial reportada para anuros (~1 km^2). Essa variação interespecífica no CT_{max} em escala local foi principalmente explicada pelos microclimas das espécies e seu uso diferencial do micro-habitat, do que pela filogenia ou massa corporal. A ontogenia teve pouco efeitos no CT_{max} de uma espécie -alvo. As HRs tiveram efeitos diferentes e independentes nas médias e variação interindividual do CT_{max} entre as espécies. Fontes de variação no CT_{max} e T_{max} tiveram efeitos no *WT* entre espécies e microhabitats, respectivamente, mas os efeitos de T_{max} foram comparativamente mais altos. Particularmente, o uso de um ST microclimático longe do T_{max} extremo levou a valores de *WT* comparáveis do que usar um ST macroclimático, e ambos conduziram diagnósticos duvidosos de vulnerabilidade ao aquecimento agudo. Nosso estudo demonstra que uma conceitualização adequada é crucial nas avaliações de vulnerabilidade baseadas em *WT*, e enfatiza que a natureza de *WT* como um traço de nível individual define limites à inferência de vulnerabilidade ao aquecimento agudo em níveis mais altos de organização biológica.

Keywords: physiological sensitivity; thermal exposure; critical thermal maximum; interindividual variation; local physiological diversity; experimental heating rates; anuran larvae; Atlantic Forest.

1. Introduction

The unprecedentedly fast, human-induced warming of the Earth's climate system is driving the planet to dangerous and irreversible scenarios for both biodiversity and humanity (Armstrong McKay et al., 2022). Since climate change ecological impacts are not homogeneous among taxa or across regions (Blowes et al., 2019; Bowler et al., 2020), determining what species and habitats are more vulnerable to climate change – an utmost goal in global change biology – is key for effective mitigation (Pinsky et al., 2019). However, and despite some synthesis efforts (Foden et al., 2019; Pacifici et al., 2015), no consensus yet exists on how to assess species vulnerability to climate change (Wheatley et al., 2017). A quick search in Google Scholar ["conceptual-framework" understanding "impact-of-climate-change" "vulnerability" (-livestock) & "biodiversity"; 31-10-2022] resulted in 2,780 publications from 2012-2022, i.e., ~24 frameworks per month. Such a proliferation of conceptual frameworks, which may disagree on criteria and final assessments, prevent researchers to provide standardized methods for managers and policymakers to base decision-making (Clusella-Trullas et al., 2021; Wheatley et al., 2017). Instead of developing a new field within global change biology (Foden et al., 2019), more interdisciplinary efforts are needed to accurately assess species vulnerability to climate change. In this context, vulnerability assessments should be "organism-centric" (Fortini & Schubert, 2017), not anthropocentric (Füssel & Klein, 2006; IPCC, 2007), and acknowledge scale dependencies in climate change ecological impacts (Bütikofer et al., 2020; Mertes & Jetz, 2018).

Warming tolerance (*WT*) is an increasingly popular index used to assess species vulnerability to warming. In its original definition (C. A. Deutsch et al., 2008), *WT* was simply the difference between the Critical Thermal Maximum (CT_{max}) and the average temperature (T_{mean}) of the habitat for a given ectothermic *organism*. *WT* users advocate that the metric relates directly aspects of physiological sensitivity and environmental exposure (sensu Williams et al., 2008, e.g., Cheng et al., 2022; Scheffers et al., 2013). Under this rationale, the *WT* concept has expanded to accommodate other measures of upper thermal limits (e.g., lethal temperatures; Marshall et al., 2015) or habitat temperature (e.g., T_{max}; Duarte et al., 2012). The simplicity of its calculation has facilitated global *WT* comparisons, which suggest that tropical/lowland species are more vulnerable to warming than temperate/high-elevation counterparts (C. A. Deutsch et al., 2008; Sentinella et al., 2020; Sunday et al., 2014). Global studies, however, have usually used only one population per species (Kim et al., 2022), neglecting that intraspecific variation exists at both sides of the *WT* equation (Clusella-Trullas et al., 2021; Sanabria et al., 2021; Villeneuve et al., 2021). Also, recent studies have shown that species at the same latitude or elevation may have contrasting *WT*, indicating that local variations in habitat (e.g., open areas vs. forest-covered) are more important determinants of *WT* than coarse-scale environmental clines (Cheng et al., 2022; Pintanel et al., 2019). Moreover, current usage of *WT* has two additional problems that remain hitherto overlooked.

The first problem is one of conceptualization. *WT* is broadly used an absolute measure of vulnerability to habitat warming, where sensitivity is solely determined by upper thermal limits. Actually, *WT* should be understood as a relative approximation of *individual* vulnerability to a specific type of warming, given but one aspect of an organism's physiological sensitivity and one aspect of its environmental exposure (Clusella-Trullas et al., 2021). For instance, the particular case $WT = CT_{max} - T_{max}$ would inform the amount of acute warming that an organism can tolerate before its performance and activity is critically compromised. Then, question, *WT* operationalization, and extent of inference are inseparable (Clusella-Trullas et al., 2021). In addition, *WT* original definition implies that this is an individual-level trait (C. A. Deutsch et al., 2008; Sandblom et al., 2016), for both components of the equation are. However, many studies, including Deutsch et al. (2008), have focused on average *WT* values as presumably representative of the *WT* for a population or species. A conceptual expansion of *WT* to populations and species would require more than just averaging data from individuals (Agudelo-Cantero & Navas, 2019), e.g., demonstrate links between *WT* and population dynamics, range limits, or extinction risk (Soroye et al., 2020). Studies using *WT* should, at the very least, provide estimates of interindividual variation around sample-wise estimates (e.g., Katzenberger et al., 2018; Sanabria et al., 2021). Nonetheless, interindividual *WT* variation *per se* is interesting and germane to the evolution of upper thermal limits (Clusella-Trullas et al., 2021; Gutiérrez-Pesquera et al., 2016), but remains completely underutilized (Agudelo-Cantero & Navas, 2019; Bennett, 1987).

The second problem is that, by definition, *WT* is sensitive to variation in both components of its equation. This issue was recently reviewed by Clusella et al. (2021), but empirical evidence remains limited and scattered. Regarding physiological sensitivity, ontogenetic (Pincebourde & Casas, 2015) and methodological (via experimental heating rates, HRs; Allen et al., 2016) effects on CT_{max} led to *WT* variation in an insect and some springtail species, respectively. Little is known whether (and how) other sources of CT_{max} variation – and potential synergies (Agudelo-Cantero & Navas, 2019) – affect *WT*, or to what extent CT_{max} related variation in *WT* differs among taxa. As for an organism's thermal exposure, this is dictated by the spatiotemporal distribution of operative temperatures in its environment (T_e;

Clusella-Trullas et al., 2021; Sunday et al., 2014). Yet, most studies have used metrics of the habitat temperature (T_{hab}) in WT calculations. Fine-scale T_{hab} measurements might approximate T_e in some contexts though, e.g., for small aquatic ectothermic animals whose body temperature is virtually isothermal with the surrounding environment (Agudelo-Cantero & Navas, 2019; Becker & Genoway, 1979). Aspects like (spatiotemporal) scale, measurement extent, and T_{hab} (or T_e) metric (e.g., T_{mean} or T_{max}) affect drastically WT estimates and inference (Garcia et al., 2019; Katzenberger et al., 2018). A less inquired factor, however, is the usage of different summary statistics for, presumably, the same metric. For instance, microclimatic "T_{max}" has been operationalized differently across studies, from the absolute (Duarte et al., 2012) or average T_{max} (Simon et al., 2015) over the whole registration period, to the median (Garcia et al., 2019) or other percentiles (e.g., 95th, 99th; Sanabria et al., 2021) of the daily T_{max} distribution. Choice criteria have not been clear though, and implications for WT values and interpretation are unknown.

A tenet for this study is that the physiological and environmental underpinnings of WT, and therefore WT itself, are individual-level traits. Hereby we investigate how variation in both WT components, specifically CT_{max} and T_{max}, influences estimates and inference of vulnerability to acute warming in an anuran assemblage. Studied populations occur within a small patch (ca. 1 km^2) of the Brazilian Atlantic Forest (Figure 1), where their larvae develop in a variety of aquatic microhabitats with markedly distinct thermal regimes (Agudelo-Cantero et al. CHAPTER 1). Capitalizing published data (Agudelo-Cantero et al. CHAPTER 1), we describe how T_{max} conditions (i.e., different summary statistics) varied within and among microhabitats and regarding the local macroclimate. We focus on T_{max} because this aspect of the thermal environment is closely related to CT_{max} in anuran larvae (Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016). We then investigate the diversity of upper thermal limits (CT_{max}) existing within our anuran assemblage, and examine the contribution of different sources of phenotypic variation (phylogenetic relatedness, microclimate, body mass, ontogeny, and HRs). Subsequently, we estimate individual *WT* as CT_{max} - T_{max}, considering sources of variation in both variables. We hypothesize a substantial physiological diversity in upper thermal limits within our study anuran assemblage, which, in conjunction with microclimate variation, configure contrasting levels of vulnerability to acute warming among species. We predict that larvae of open-area species are the most heat tolerant, yet most vulnerable to acute warming, relative to forest-dwelling counterparts, because the former experience microclimatic temperatures closer to their upper thermal limits.

2. Material and methods

2.1. *Study area and system*

Our study area is located around the headquarters of the Intervales State Park (ISP; 24º31’03’’ – 24º11’’36’ S and 48º31’22’’ e 48º3’13’’W, 800 – 900 m a.s.l.), a reserve located in the São Paulo State, southeastern Brazil, (Figure 1; Furlan & Leite, 2008b). The ISP is within the domain of the Atlantic Forest, a highly fragmented biodiversity hotspot than extends from northeastern Brazil over the Atlantic coast of the country, and reaches northeastern portions of both Paraguay and Argentina (Morellato & Haddad, 2000; Sandro Menezes Silva, 2017). Vegetation in our study site is mainly characterized by young secondary forests given anthropic influences, but old secondary forests are well preserved in some areas away the ISP headquarters (Carvalho et al., 2002). Local macroclimate (historical records of air temperature and precipitation from 1990-2016 retrieved from the ISP weather station; Figure 1c) in our study site follows regional patterns (Morellato et al., 2000), with a cold dry season from May to September and a warm wet season from November to March (Bertoluci & Rodrigues, 2002; Furlan & Leite, 2008a).

Regarding anuran diversity, the ISP management plan (Furlan & Leite, 2008a) reported 66 species occurring within the park limits (50% endemic to the Atlantic Forest). Forty-seven species were documented in the 1990s over only 3 km^2 around the ISP headquarters (Bertoluci, 1998; Bertoluci & Rodrigues, 2002). We observed 14 species in total within our study area during short visits from 2014-2019 (Agudelo-Cantero et al. CHAPTER 1). Anyhow, most anuran species in the ISP lay aquatic eggs that develop into exotrophic aquatic tadpoles, whereas few species exhibit more specialized reproductive modes (e.g., terrestrial eggs with direct development or semiterrestrial tadpoles). Reproductive activity for most anurans in the ISP occurs during the warm wet season (Bertoluci, 1998), and species take advantage of a diversity of aquatic microhabitats that differ in both structural and thermal characteristics (Bertoluci & Rodrigues, 2002; Agudelo-Cantero et al. CHAPTER 1).

2.2. *Samplings and field experiments*

Fieldwork was conducted during 14-23 November 2017 and 03-10 February 2019. We observed breeding adults (e.g., calling males, amplexus) in twelve water bodies of distinct characteristics (size, temporality, water motion, canopy cover), but larvae were present only in five water bodies (Figure 2). Water temperatures for all water bodies were registered as part of a parallel study (Agudelo-Cantero et al. CHAPTER 1), via HOBO Pendant dataloggers (Onset Computer Corporation, Bourne, MA). HOBOs were set to record water temperature at 10-min intervals, and then deployed at the bottom of water bodies for this is their coolest area (Newman, 1989; Woods et al., 2015).

We collected anuran larvae (hereafter tadpoles) only for species with more than 10 individuals (7 species; Bufonidae: 1 spp., Hylidae: 4 spp., Hylodidae: 1 spp., Odontophrynidae: 1 spp.; Figure 3). We also collected dragonfly naiads of the species *Anax concolor* (Aeshnidae) in one water body in which we found tadpoles for two anuran species. We sampled water bodies between 0530-0630 local time, and transported individuals in plastic boxes with dechlorinated water to a field lab located within our study site (Figure 1c). Water temperature at these containers was maintained at ~20°C, close to water bodies' temperature at sampling times (Agudelo-Cantero et al. CHAPTER 1). We kept individuals in these conditions for ca. 1 hour before experiments and observed their morphology and behavior during that time. No further pre-treatment or environmental control was conducted prior to experiments in order to determine field CT_{max}, i.e., immediate responses of freshly-collected organisms (Hoffmann & Sgrò, 2018). Unless otherwise stated (see below), we tested tadpoles in Gosner stages 25-41 (Gosner, 1960), a developmental window when CT_{max} is presumably stable before declining sharply at the metamorphic climax, according to the few species tested (reviewed by Bodensteiner et al., 2021).

All experiments were conducted between 0800-1300 local time. We employed the Hutchison's dynamic method (Hutchison, 1961) and used the setup designed by Agudelo-Cantero & Navas (2019) to measure field CT_{max} at two constant heating rates (HRs): 1°C/min, a rapid HR to estimate a baseline level of CT_{max}; and 0.1°C/min, the maximum natural HR registered among microhabitats of larval anurans at the study area (Agudelo-Cantero et al. CHAPTER 1), to mimic a daily heating scenario in which water temperature peaks between 1200-1300. Briefly, we placed animals individually inside plastic containers with dechlorinated water. Then, we assigned individuals randomly to one HR and transferred containers to temperature-controlled baths 10 minutes before starting experiments. Initial water temperature was 20°C (ca. overall average temperature among sampled water bodies), and then was constantly increased by hand modification on bath settings according to a given HR. We deployed a HOBO into one control container (same water volume but no animal) to register water temperature throughout all experiments at 1-min intervals to measure actual HRs.

We continuously inspected shifts in animal behavior with temperature increases, while monitoring water temperature next to individuals using a fast-reading thermometer. At a certain temperature, swimming activity for both tadpoles and dragonfly naiads became disorganized, with cycles of abrupt bouts followed by short inactivity. We did not observe spasms in all individuals, and loss of righting response was ambiguous for some, so we ended experiments when animals did not react after five consecutive taps with the thermometer (Agudelo-Cantero & Navas, 2019). At this moment we registered water temperature besides the individual as its field CT_{max}, and then transferred it immediately into a plastic container with cool water (~20°C) for recovery. We considered only the data of animals that displayed normal behavior 24 h after the tests. After that time, we euthanized individuals in 5% lidocaine and then fixed them in 10% formalin. Samples were transported to the Laboratory of Ecophysiology and Evolutionary Physiology, IB-USP, where we confirmed taxonomic identification and measured body mass with an analytical balance to the nearest 0.001 g.

2.3. *Data analyses*

All data analyses and visualization were performed in the R programming environment (version 4.2.1; R Core Team, 2022), and the data and code supporting the results of this study will be openly available in a public repository after publication.

2.3.1. The thermal environment

Data collected by HOBOs during our samplings were analyzed in a purposely parallel study (Agudelo-Cantero et al. CHAPTER 1). Here we explored HOBOs data and provide new exploratory analyses for the distribution of water temperatures realized by our studied community, particularly the maximum temperatures (T_{max}). Thermal environment data was also used in subsequent analyses of CT_{max} variation and for *WT* calculations (see below).

2.3.2. Field Critical Thermal Maximum (CT_{max})

We conducted three independent analyses to investigate the contribution of historic (phylogeny), extrinsic (microclimate), intrinsic (ontogeny, body mass), and methodological

(HRs) factors to the community-level variation in field CT_{max}. *Analysis 1* assessed to what extent interspecific differences in field CT_{max} (slow HR, anurans only) were related to species evolutionary history vs. the most recent thermal experience. We could not include phylogenetic relatedness explicitly in this analysis though, given the low number of species ($N = 7$) and supraspecific representation (6 genus, 4 families) in our experimental design (Blomberg et al., 2003). Nevertheless, we incorporated phylogenetic considerations in our inference by using a topology based on a tree retrieved from TimeTree5 (Kumar et al., 2017). One studied species (*Scinax rizibilis*) was not included in the TimeTree5 database, so we applied the known-sister taxon approach (Gutiérrez-Pesquera et al., 2016; Simon et al., 2015) and used the position of the closest relative (*S. catharinae*; Faivovich et al., 2005) to determine the final topology (Figure 3).

Regarding the thermal environment, we calculated relevant summary statistics for the microclimatic temperatures experienced by tadpoles 24 – 48 h before experiments, as follows: *levee*, T_{max}, T_{min}, dT (T_{max} - T_{min}), dCV%, and the natural hating rate HR_{eco} (calculated as dT/t, where t was time in min from T_{min} to T_{max}). Likewise, we used registers of the local weather station of the ISP during the same time period to calculate the following summary statistics for the local macroclimatic temperatures: *MAX*, *MIN*, and *DT* (*MAX* - *MIN*). We averaged summary statistics over the considered time period and used them separately to test the relationship between a particular aspect of the thermal environment (micro or macro) and species field CT_{max} at a time. We then fitted multiple competing generalized linear models via maximum likelihood (ML) parameter estimation (Pinheiro & Bates, 2000), where field CT_{max} was our response variable and fixed effects were *Species* and a given *thermal environment predictor*. *Body mass* was also included in all models as covariate (Table S1). We ranked models based on their *AICc* values (second order Akaike information criterion; Akaike, 1974; Sugiura, 1978) and selected those with $\Delta AICc < 2.0$ (Burnham et al., 2011; Burnham &

Anderson, 2002). We also compared the relative support of models, given the data, by computing *AICc* weights (w_i), as well as evidence ratios ($ER = w_1/w_n$) between the top-ranked model and alternative ones (Burnham & Anderson, 2002). This model selection procedure was applied in subsequent analyses.

In *analysis 2*, we tested whether (and how) HRs affected the field CT_{max} of tadpoles. We contrasted part of the dataset used in analysis 1 with comparable CT_{max} data obtained for species tested at the rapid HR ($N = 5$ species). We fitted three general linear models hypothesizing that (a) species were differentially sensitive to HR effects, (b) HR effects were consistent for all species, or simply that (c) species were not sensitive to HRs (Table S1). We did not include *body mass* as covariate in any model here (see *Results*). Afterwards, we ran a pairwise interaction test via the R “phia” package (de Rosario-Martinez, 2015), controlling the false discovery rate to adjust *P-values* (Benjamini & Hochberg, 1995), to determine how each anuran species was affected by HRs. We also tested whether anuran species responded differently to HRs in terms of their interindividual variation of field CT_{max} via Levene’s test ($\alpha = 0.05$).

Analysis 3 evaluated whether the field CT_{max} of *Rhinella icterica* changed along its ontogeny, and whether morphologically distinct ontogenetic stages were differentially sensitive to HR effects. For this analysis we used data from a second sample of *R. icterica* tadpoles found in a different water body and exhibiting wider ontogenetic variation than the first sample included in *analysis 1* (Figure 2). We fitted generalized additive models (Gaussian fit, ML parameter estimation) to field CT_{max} data, with *Gosner stage* as a fixed, ordinal predictor for ontogenetic change using the approach of Gertheiss et al. (2022). Competing models hypothesized that (a) ontogenetic shifts in field CT_{max} varied with HRs, (b) field CT_{max} of *R. icterica* changed through ontogeny independently of HRs, or (c) there was no ontogenetic shift in the field CT_{max} of *R. icterica* (Table S1).

Finally, *analysis 4* assessed the variation in field CT_{max} between trophic levels in our studied community. We focused on anuran species developing in the open temporary pond (*Dendropsophus minutus* and sample 1 of *R. icterica*) and contrasted their field CT_{max} (data from *analysis 1*) with comparable data gathered from dragonfly naiads (Figure 2). We fitted generalized linear models (ML estimation) and tested whether (a) species differed in both sample averages and interindividual variation of field CT_{max}, (b) species differed only in mean field CT_{max} responses, or (c) species did not differ in field CT_{max} (Table S1).

2.3.3. Warming Tolerance (*WT*)

We investigated how accounting for sources of field CT_{max} variation or different summary statistics for T_{max} affected final *WT* estimates and inference. We first assessed T_{max}-related variation in *WT*. We fixed the CT_{max} component by using only data collected at the slow rate (0.1°C/min) and included all species and samples in the community. We then estimated individual *WT* (and sample averages) using either micro- or macroclimatic summary statistics for T_{max}: (a) the median (*dmax*) or (b) the 95th percentile (*max.95*) of the daily T_{max} registered in water bodies, or (c) the 95th percentile (*MAX.95*) of the daily T_{MAX} registered by the ISP weather station. Since T_{max}-related effects in *WT* just reflect raw differences among T_{max} indices, no formal analysis was needed here. Thus, we inspected and described these effects visually.

Afterwards, we scrutinized CT_{max}-related variation in *WT*. To fix the T_{max} component, we used only *max.95* values in *WT* calculations. Then we computed *WT* for all individuals and samples, using field CT_{max} data measured at different HRs (N = 5 species) and in different ontogenetic stages (for *R. icterica*). We showed these effects graphically. Furthermore, we used *WT* data from slow-rate CT_{max} to test the hypothesis that anuran species differed in both average (sample-wise) and interindividual variation of vulnerability to acute warming vs. an alternative hypothesis considering only species variation in average vulnerability (Table S1).

3. Results

3.1. *The thermal environment*

Overall, water bodies in open areas were warmer, more variable, and more extreme than those inside the forest and covered by canopy (Table 1). Interestingly, open water bodies exhibited the highest microclimatic temperatures but in a sporadic fashion (see below), as inferred from their broad, right-skewed temperature distributions (Figure 2). In contrast, canopy-covered water bodies were rather cool and displayed narrower, more symmetric temperature distributions centered around average values 18.5-20.8°C. Within areas, the small and shallow temporary pond was more thermally variable and extreme than the bigger and deeper permanent pond located only ca. 80 m away (Figure 1). Inside the forest, the stream was the most constant and coolest water body over our study period.

A further inspection to the distribution of daily microclimatic T_{max} registered over our study period is also given in Table 1. Clearly, variability of daily T_{max} was much higher in open water bodies than in forest counterparts. Main daily T_{max} differences concentrate between the two-upper quartiles of the distributions, where open water bodies exhibited temperatures above 30°C and canopy-covered water bodies did not. Yet, water temperatures equal to *dmax* (dashed blue line, Figure 2) were not uncommon and occurred for 25.5-115.8 h over our study period. On the contrary, water temperatures equal to *max.95* (dashed red line, Figure 2) were indeed infrequent in all water bodies, particularly at open areas. In relation to the local macroclimatic temperature, only the open permanent pond displayed daily T_{max} conditions similar to those registered by the ISP weather station (T_{MAX}).

3.2. *Field CT_{max}*

3.2.1. Phylogeny vs. thermal environment

Our studied species differed in their mean field, slow-rate CT_{max} (Table 2), and this interspecific variation was not simply associated to species' phylogenetic relationships (Figure 3). Instead, CT_{max} evolution among our studied species reflects more their microhabitat use, for species inhabiting the same kind of water body had comparable average CT_{max} despite their phylogenetic distance (Figure 4a). Furthermore, average CT_{max} for species was positively related to both microclimatic T_{mean} and T_{max} that tadpoles experienced during the last 48 h prior to experiments (Figure 4b; Table S1). Thus, three species groups, with correlated thermal physiology and microhabitat use, compose our studied anuran assemblage (Figure S1): (a) open-pond developing species (*R. icterica* and *D. minutus*), with the highest field CT_{max} and inhabiting the hottest microhabitats; (b) forest-pond developing species (*B. faber, D. giesleri, S. rizibilis, P. boiei*), with intermediate field CT_{max} and dwelling in cool water bodies; and (c) a stream-specialist species (*C. caramaschii*), with the lowest field CT_{max} and occupying the coolest and most constant microhabitat. Body mass was highly variable within species, but it was not related to interspecific variation in field CT_{max}.

3.2.2. Experimental heating rates (HRs)

Anuran species responded differently to HRs in terms of average and interindividual variation of field CT_{max} (Figure 4c; Table 1; Table S1). Regarding mean effects, *Crossodactylus caramaschii* was the most sensitive species (pairwise interaction test, $F_{1,175} = 65.43$, $P < 0.01$), for its slow-rate CT_{max} (0.1°C/min) was on average 1.5°C lower relative to its rapid-rate CT_{max} (1°C/min). No other species was significantly affected by HRs (pairwise interaction tests, $F_{1,175} = 0.267 - 1.56$, $P > 0.05$), yet two patterns are worth mentioning. Tadpoles of *R. icterica* (1st sample) and *S. rizibilis* were largely insensitive to HR effects (average difference between rapid-rate CT_{max} vs. slow-rate CT_{max} = 0.2°C). Also, *D. minutus* tadpoles increased their average slow-rate CT_{max} relative to their rapid-rate CT_{max} (0.4°C). Regarding interindividual variation, all species displayed less variable slow-rate CT_{max} compared to their rapid-rate CT_{max} (Levene's test, $F_{9,175} = 4.91$, $P < 0.01$; Figure S2). The magnitude of this effect varied among species, though, with *C. caramaschii* and *P. boiei* as the most sensitive species (> 40% reduction of interindividual variation of CT_{max} at the slow rate). Interestingly, HR effects on average and variance of CT_{max} seemed independent across species.

3.2.3. Ontogeny

The field CT_{max} of *R. icterica* (2nd sample, Figure 2) varied little across ontogeny, and HR effects were negligible (-0.2°C at the slow vs. rapid HR; Table S1). The ontogenetic shift was better described by a left-skewed curve (Figure 4d): field CT_{max} increased steadily +0.4°C from early ontogenetic stages (Gosner 31) until right before the metamorphic climax (Gosner 41), then decreased sharply -0.5°C until the end of metamorphosis (Gosner 45). When field CT_{max} data at both HRs are pooled and similar Gosner stages (29-31) are compared between *R. icterica* samples, we observed that variation in the developmental environment drove a much larger effect on field CT_{max} than ontogeny. Tadpoles from the 2nd sample, collected in the open permanent pond, were on average 1.4°C less heat tolerant than conspecifics from the 1st sample collected in the open temporary pond.

3.2.4. Tadpoles vs. predator

Regarding field CT_{max} across trophic levels (open temporary pond, Figure 2), species differed in both sample averages and interindividual variation (Table S1). Dragonfly naiads (*A. concolor*) were on average 2.0-2.7°C more heat tolerant than syntopic tadpoles (*R. icterica* and *D. minutus* respectively; Table 2). On the other hand, *D. minutus* tadpoles were the most variable in their field CT_{max} relative to the other species, whose interindividual variation seemed driven mostly by few sensitive individuals (*n* = 1 for *R. icterica*, *n* = 2 for dragonfly naiads; Figure S3). When these individuals were excluded, mean CT_{max} of dragonfly naiads increased (0.2°C) and the standard deviation decreased (0.4°C, or 66% reduction relative to original sample), but effects on *R. icterica* were negligible.

3.3. *Warming Tolerance* (*WT*)

3.3.1. T_{max}-related variation

The choice of T_{max} summary statistics had great impacts on both *WT* estimation and inference thereof, but ultimate consequences varied among microhabitats (Figure 5). For instance, under *max.95* the most vulnerable species to acute warming (*WT* close to 0) were those inhabiting the open temporary pond, whereas forest-dwelling species were the least vulnerable (higher *WT*; Table 2). But under *dmax*, *WT* would inform a rather low vulnerability to acute warming for the entire community (*WT* > 10°C), for *WT* values (individual and sample averages) were overestimated relative to *max.95*-derived *WT*. Such an effect was higher for open-area species (+9.9°C) than for forest-dwelling species (+2.1°C), given *max.95 – dmax* (Table 1). On the other hand, using *MAX.95* would inform that forest-dwelling species were the most vulnerable, for their *WT* was underestimated by -8.5 to -11.8°C relative to *max.95*-derived *WT* (given *max.95* - *MAX.95*; Table 1). Still under *MAX.95*, species from the open temporary pond would be the least vulnerable, for their *WT* was overestimated by +7.2°C.

3.3.2. CT_{max}-related variation

Species clearly differed in both their sample averages and interindividual variation of *WT* (Table 2; Figure 5; Table S1). Mirroring CT_{max} patterns, interspecific *WT* variation was not closely associated to species phylogenetic relationships but to microhabitat association (Figure 6a). In its most extreme case, such a phylogenetic independence/microhabitat association of *WT* is exemplified by *Dendropsophus* species. While *D. giesleri* – a forest pond-dwelling species – is on average the least vulnerable species of the assemblage to acute warming, *D. minutus* – an open pond-dwelling species – is the most vulnerable.

Naturally, mean and variances of *WT* inherited effects from methodological (HRs) and intrinsic (ontogeny) sources of CT_{max} variation (Table 2). Overall, slow HRs led to lower *WT* averages in all species but *D. minutus* (Figure 6b). Likewise, interindividual *WT* variation decreased for all species when estimated from slow-rate CT_{max} data. Interestingly, three *D. minutus* tadpoles (8% of the sample) tested at the rapid HR exhibited negative *WT* values ranging -0.43 to -0.03 (i.e., $CT_{max} < max.95$). For comparison, the most vulnerable *D. minutus* tadpole tested at the slow HR exhibited a *WT* value of +0.46°C ($CT_{max} > max.95$). On the other hand, *R. icterica* tadpoles at different ontogenetic stages (2nd sample) varied little in their average or individual vulnerability to acute warming (0.5°C mean *WT* difference between Gosner stages 41 vs 45; *WT* range: +7.6 to +9.3°C; Figure 6c). Yet, these *R. icterica* tadpoles, developing at the open permanent pond, were on average 6.2°C less vulnerable to acute warming (higher *WT*) than counterparts developing at the warmer and more extreme open permanent pond (Figure 6c).

4. Discussion

Almost 80 years ago, Cowles & Bogert (1944) conceived the CT_{max} as the ecological [upper] lethal temperature, for an ectothermic animal that has collapsed by heat would be more susceptible to predation and would promptly die if heating continues. Physiologically speaking, CT_{max} is nearly fatal for many insects but not for ectothermic tetrapods (Clusella-Trullas et al., 2021), which usually recover from a critical thermal exposure. Although motile ectothermic organisms would tend to avoid reaching CT_{max} (Nelson et al., 1984; but see Navas et al., 2022), this trait becomes preponderant when opportunities to thermoregulate behaviorally are limited, e.g., sessile/developing forms, shallow aquatic environments

(Bodensteiner et al., 2021; Gutiérrez-Pesquera et al., 2016). As microhabitats warm up due to global warming, estimating the proximity between CT_{max} and microclimatic T_{max}, i.e., warming tolerance (*WT*), seems a quick and practical method to identify populations that might be vulnerable to acute warming. Nonetheless, *WT* users are to be aware of the nature of this metric, the traits involved in its calculation, and the extent of inference, in order to inform accurately policy makers. By using a local case study, we demonstrate that variation in both components of *WT*, particularly in the operationalization of the environmental exposure, resulted in fundamentally distinct assessments of vulnerability to acute warming in an anuran assemblage.

4.1. T_{max}-*related variation*

The use of different summary statistics for T_{max} in *WT* calculations had large impacts on both individual and sample average estimates, as well as assessments about vulnerability to acute warming in our study community (Figure 5). Previous studies addressing impacts of variation at the thermal exposure component of *WT* have concentrated on the dichotomy “macroclimate vs. microclimate” (e.g., Curtis et al., 2016; Katzenberger et al., 2018; Scheffers et al., 2013). This is not a minor problem, because misunderstanding the spatial and temporal scales at which individual organisms sense their environment leads to erroneous (and sometimes problematic) diagnosis of vulnerability (Anderson et al., 2022; Pincebourde & Casas, 2015). But, even if microclimatic (or operative) temperatures are available, two intertwined questions remain: which aspect(s) of the thermal environment (e.g., T_{mean} or T_{max}) should be considered in *WT* calculations, and what summary statistics thereof (e.g., absolute T_{max}, daily average T_{max}, etc.) should be employed?

The answer to the first question, according to Clusella-Trulas et al. (2021), is that a range of options are possible and can provide distinct ecological insights. Although we agree, this does not mean that all aspects of the thermal environment matter for CT_{max}. Our data suggest that the mean CT_{max} of anuran species was positively related to both microclimatic T_{mean} and T_{max} experienced by individuals 48h before experiments. Yet, T_{max} is likely more relevant for CT_{max} evolution in anuran larvae (Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016) and other ectothermic animals (Chown et al., 2010; Clusella-Trullas et al., 2011) than T_{mean}. The second question, however, has not received proper attention nor has obvious answer. For our study species, using either the median daily microclimatic T_{max} (*dmax*, suggested by Garcia et al., 2019) or the 95th percentile of daily macroclimatic T_{MAX} (*MAX.95*) led to comparable effect sizes (> 7°C differences in *WT*), yet microhabitat-specific, relative to the 95th percentile of daily microclimatic T_{max} (*max.95*). Moreover, both *dmax* and *MAX.95* failed to capture the proximity between extreme T_{max} and individual CT_{max} (Figure 2), the essence of *WT*, and therefore led to dubious diagnosis of vulnerability to acute warming. Using *max.95*, which represented better extreme T_{max} (Table 1), resulted in estimates closer to *WT* definition, and so in more accurate assessments of vulnerability. Since extreme quantiles for microclimatic T_{max} may explain better thermal differences among microhabitats than central quantiles (Camacho et al., 2015), we suggest using the former in *WT* calculations.

4.2. *CT_{max}-related variation*

In agreement with our hypothesis, we corroborated that our study anuran assemblage harbors a high diversity of upper thermal limits (individual CT_{max} range = 32.3 – 42.4 °C; Figure 2). This physiological diversity was mainly explained, among all sources of CT_{max} variation considered, by species differences in developing microhabitat and realized microclimatic T_{max} (Figure 4a, b). Despite the few species we compared, our results further suggest a minor role of phylogenetic relatedness in explaining species differences in CT_{max}. The CT_{max} of anuran larvae generally shows phylogenetic signal – closely related species have similar CT_{max} under a Brownian motion evolutionary process than distantly related species – (Cheng et al., 2022; Duarte et al., 2012; Gutiérrez-Pesquera et al., 2016; but see Madalozzo, 2018), but evidence for the CT_{max} of adult frogs is mixed (Carilo Filho et al., 2021; Pintanel et al., 2019; von May et al., 2017, 2019). Nevertheless, these studies indicate that CT_{max} evolution in anurans is mostly driven by microhabitat use and the concomitant microclimatic exposure (Bodensteiner et al., 2021), factors that explain diversification rates among families (Moen & Wiens, 2017). Understanding CT_{max} evolution, alongside with thermal environment information, may shed light on phylogenetic patterns of current vulnerability to acute warming among taxa (e.g., Figure 6a), with potential benefits for *WT*-based assessments. For instance, if *WT* is conserved among species, one might approximate the *WT* of a species from a close relative (Clusella-Trullas et al., 2021). On the other hand, if *WT* is labile and largely determined by microhabitat use, the *WT* of one species might broadly inform the *WT* of ecologically similar counterparts (e.g., syntopic species). In any case, evolutionary considerations in *WT*-based assessments could save time, money, and effort.

Compared with microhabitat-related influences, effects of intrinsic (body mass, ontogeny) and methodological (HRs) factors on CT_{max} (and therefore *WT*) were smaller but still relevant (Figure 4c, d; Figure 6b, c). Body mass did not explain interspecific CT_{max} variation, yet we do not discard that this variable may impact intraspecific CT_{max} variation. Detecting size effects on CT_{max} within species requires, though, larger samples than those we used in this study (Agudelo-Cantero & Navas, 2019; Ribeiro et al., 2012). Nevertheless, size effects on CT_{max} may either reflect physiological variation among individuals of different sizes in a population, or arise from methodological artifacts acting in connection with thermal inertia (Agudelo-Cantero & Navas, 2019; Ribeiro et al., 2012). Body size also relates to how animals perceive T_{max} in space and time, for it constraints the spatial dimension of accessible microhabitats and interacts, via thermal inertia, with rates of thermal change (Agudelo-

Cantero & Navas, 2019; Potter et al., 2013). Thus, size-related differences in *WT* can be expected within populations, particularly in size-variable taxa. Since smaller sizes are predicted under climate warming, mainly for aquatic organisms (Deutsch et al., 2022; Forster et al., 2012), understanding size differences in individual vulnerability to acute warming is critical.

Effects of experimental heating rates (HRs) were small in terms of mean CT_{max}, considerable in terms of interindividual variation and, in both cases, largely species-specific. Diverse CT_{max} responses can be expected among and within taxa regarding influences of HRs, given implications of time of exposure on individual physiology (Rezende et al., 2011, 2014). Slow HRs (i.e., long thermal exposures), on one hand, may exacerbate metabolic costs and decrease CT_{max}, or elicit physiological adjustments (e.g., thermal hardening) that increase it (Rezende et al., 2011; Terblanche et al., 2011). Rapid HRs, on the other hand, would filter out both costs and adjustment possibilities, capturing presumably a baseline CT_{max} response (Agudelo-Cantero & Navas, 2019; Ribeiro et al., 2012). In our study anuran assemblage, slow HRs seemed to have posed majorly costs on the CT_{max} of most species but *D. minutus*, where a few individuals might have experienced a beneficial physiological adjustment. Our results are similar to a previous study that showed a decrease in interindividual CT_{max} variation at slow HRs in larvae of two anuran species (Agudelo-Cantero & Navas, 2019), albeit it is still premature to suggest a general pattern given the few species examined. Nevertheless, CT_{max} dependence on HRs has been long documented in a variety of ectothermic lineages (Agudelo-Cantero & Navas, 2019; Chown et al., 2009; Ribeiro et al., 2012; Terblanche et al., 2007; Vinagre et al., 2015), so *WT*-based assessments should recognize this source of variation. Moreover, given the intended ecological significance of *WT*, CT_{max} data necessary in this context should be estimated under an ecological HR that matters for the focal taxon, i.e., based on natural HRs experienced by individuals (Allen et al., 2016).

In *R. icterica* tadpoles, CT_{max} changed less than 1°C throughout metamorphosis (and so *WT*), following a left-skewed curve (Figure 4d). Although the number of individuals per Gosner stage was limited, particularly at both early and late stages, our observations agree with the notion that heat tolerance of anurans matures through metamorphosis and declines at the metamorphic climax (reviewed by Bodensteiner et al., 2021). It is likely that the heat tolerance of anurans changes in concert with the realized thermal environment during life history transitions, with potential carry-over effects from previous to subsequent stages (Bodensteiner et al., 2021). For instance, lethal temperatures increased progressively from embryos to tadpoles in several species (see Figure 1 in Turriago et al., 2015), but exceptions exist (Herreid & Kinney, 1967; Kuramoto, 1978). Likewise, CT_{max} reduction at the metamorphic climax may characterize CT_{max} levels of juveniles in some species (e.g., *Rana temporaria*; Enriquez-Urzelai et al., 2019), but not in others (e.g., *Rhinella granulosa*; Navas et al., 2007; Simon et al., 2015). Time of exposure is also a relevant factor, for sensitive stages may retain physiological capacity to cope with (and even adjust to) certain types of thermal challenges (e.g., slow HRs; Agudelo-Cantero et al., 2019). The complexity of responses, as well as diversity among taxa, calls attention to potential changes in *WT* in anurans and other ectothermic animals through ontogeny, given ontogenetic shifts in CT_{max}, T_{max}, or both (Pincebourde & Casas, 2015). Ontogenetic variation in *WT*, via CT_{max}, was small in our study species, but clearly developing in a warmer microhabitat decreased dramatically *WT* in *R. icterica* (> 6°C; Figure 6c). Thus, adult anurans may constraint the *WT* of their offspring via oviposition-site choice within populations, even at microgeographic scales (see below).

4.3. *Further considerations*

The diversity of CT_{max} observed in our study anuran assemblage occurs within a subtropical forest patch of only 1 km^2 (Figure 1). Within-community variation in heat tolerance has been reported mainly for invertebrates (Baudier et al., 2018; Compton et al., 2007; Kaspari et al., 2015), with the most extreme case of mini arthropods inhabiting the same host plant (Pincebourde & Casas, 2019). Few equivalent reports exist for anuran assemblages occurring in areas as small as 5 km^2 (Pintanel et al., 2022; von May et al., 2019), but clearly interspecific variation in heat tolerance may occur at smaller spatial scales. In all cases, ours included, local variation in heat tolerance is linked to both spatial microclimatic thermal heterogeneity and distinct microhabitat use among species. Particularly, the most heat tolerant species usually occupy the warmest microhabitats, which paradoxically turn them the most vulnerable to acute heating within communities. In our study assemblage, this is the case of species developing in open-area ponds, particularly temporary, relative to water bodies under canopy (Figure 5). Higher vulnerability to acute warming in open-area vs. forest-dwelling species seems a general pattern in anuran larvae across latitude or elevation (Cheng et al., 2022; Pintanel et al., 2019, 2022). Acute warming may further affect the most vulnerable tadpoles in our study assemblage (*D. minutus* and *R. icterica*) indirectly. By being less heat tolerant than a natural predator (dragonfly naiads), tadpoles' locomotor performance may be impaired by near-critical high temperatures before predators are affected, and so become susceptible to predation (Katzenberger et al., 2021; Pintanel et al., 2021).

Collectively, evidence from local-scale studies has multiple implications for our understanding of heat tolerance in ectothermic animals, as well as their vulnerability to acute warming. First, heat tolerance can vary among taxa at microgeographic scales, sometimes independently of phylogeny (Carilo Filho et al., 2021; Stillman, 2002). This fact challenges a long-established notion of phylogenetic conservatism in heat tolerance among ectothermic animals, derived from observations across macroclimatic gradients (Bozinovic et al., 2014; Gaston et al., 2009; and citations therein). Second, microclimatic thermal variability may drive local variation in heat tolerance among species with distinct natural history, even to a greater extent than macroclimatic gradients (Kaspari et al., 2015; this study vs. Gutiérrez-

Pesquera et al., 2016). The above supports the idea that Janzen's climatic variability hypothesis (Janzen, 1967; Stevens, 1989), originally proposed to link thermal tolerance and species ranges to macroclimatic thermal variation, may be extended to microclimates and within-habitat occupation (Baudier et al., 2018; Klinges & Scheffers, 2021). Third, local diversity in microclimates, thermal physiology, and natural history may jointly set a mosaic of vulnerabilities to acute warming (*sensu WT*) within assemblages or communities (Carilo Filho et al., 2021; Pincebourde & Casas, 2019; von May et al., 2019). Thus, broad *WT* comparisons between whole communities (e.g., tropical vs. temperate) may be rather inadequate for they homogenize local-scale variation in *WT* components. If commonalities driving large-scale *WT* patterns do exist among taxa, they shall relate to microscale aspects like habitat differences (e.g., open vs. forest-covered microhabitats of anurans, see above), instead of macroscale factors like latitude or elevation.

A final comment relates to the importance of recognizing interindividual variation in *WT*-based vulnerability assessments. It was long established in evolutionary theory that phenotypic variation among individuals is the raw substrate for natural selection (Darwin, 1859). Nevertheless, emphasizing "the mean phenotype" of the population over the individual data used to calculate it ("the tyranny of the Golden Mean"; Bennet, 1987) has been a common practice in global change biology (Guscelli et al., 2019). Among all papers published in *Global Change Biology* so far (> 7,1 K; 1995-2022), 0.3% (22) mentioned interindividual variation, +0.3% (20) actually investigated it, and +0.1% (9) simply controlled it to estimate mean effects (Table S2). Likewise, countless of studies using *WT* have focused entirely on mean values, ignoring the individual level of this metric. For instance, a typical plot of *WT* among taxa usually contains one average value per population/species (e.g., Figure 2 in Katzenberger et al., 2018) and, at best, a measure of uncertainty around it (e.g., Figure 6 in Sanabria et al., 2021, or Figure 3b in Cheng et al., 2022). A measure of dispersion (e.g., standard variation, range), or the raw *WT* individual data, are rarely (if ever) presented. Our results showed that species differed in both average and interindividual variance of *WT* (Figure 5). Ignoring the latter would lead to overestimate the vulnerability to acute warming of populations by missing the most sensitive individuals on the left side of the distribution.

5. Conclusions

The Warming Tolerance (*WT*) index estimates how close individual organisms live to their upper thermal limits (CT_{max}), providing useful insights into their vulnerability to acute habitat warming. Its mean value offers a good starting point to propose hypotheses about the population-level vulnerability, and so it should not be taken as final. This is because, being calculated from individual-level traits, *WT* is subject to sources of variation on its components and shows interindividual variation. By integrating different sources of variation in both *WT* components in a single field study, we demonstrated that *WT*-based diagnosis of vulnerability to acute warming depends on how this index is conceptualized and applied. For our study species, using a microclimatic summary statistic far from extreme T_{max} led to comparable *WT* values than a macroclimatic summary statistic, indicating that relying on microclimatic data *per se* is not enough and that appropriate operationalization of the thermal environment is key. Although effect sizes of CT_{max}-related variation on *WT* estimates were comparatively smaller, they are by no means irrelevant. Experimental heating rates, for instance, had different and independent effects on averages and interindividual variance of CT_{max} across species, and so their impacts on *WT* were both species- and context-specific as well. Ontogeny had little impacts on both CT_{max} and *WT* of our target species, but current evidence suggests this case may be an exception for anurans. If body size drives inter-individual differences in *WT* remains an open question. Interestingly, our data show that ectothermic vertebrates can exhibit substantial interspecific variation in heat tolerance over microgeographic scales. Then, organisms' vulnerability to acute warming should not be expected to be homogeneous within communities, even in those previously suggested as the most vulnerable in the tropics.

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Ethics statement

Access to the Intervales State Park was provided by the Forest Institute of the São Paulo state (SMA Process No. 260108 – 000.372/2015). Animal samplings were made under a SISBIO license (14836-1) from the Chico Mendes Institute for Biodiversity Conservation, Ministry of the Environment, Brazil. All rearing and experimental procedures were reviewed and approved by the Animal Ethics Committee (license 269/2016) of the Institute of Biosciences, University of São Paulo. Access to the genetic heritage was registered in the Brazilian Genetic Heritage Management Council (CGEN) under a SISGEN register (A66D1F4).

Conflict of interest

The authors declare no competing interests.

Author contributions

Gustavo A. Agudelo-Cantero: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing, review & editing. **Pedro L. Atencia-Gándara:** Methodology, Writing, review & editing. **Fernando R. Gomes:** Conceptualization, Supervision, Writing, review & editing. **Carlos A. Navas:** Conceptualization, Funding acquisition, Supervision, Writing, review & editing.

Data availability

The data and the code supporting the findings of this study will be openly available in a public repository after publication.

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TABLES

Table 1. Summary statistics for the temperature of water bodies (°C), as registered by HOBOs. Available counterparts for temperature data retrieved from the weather station of the ISP are also given. *N* informs both the total number of data recordings (10-min intervals) and the corresponding number of consecutive days of recordings in brackets. *Min* and *Max* are the absolute minimum and maximum temperatures of the time series, and *Range* is the difference between these two. The 0th (*max.0*), 25th (*max.25*), 50th (*dmax*), 75th (*max.75*), and 95th (*max.95*) percentiles of the daily maximum temperatures are also provided. *p.dmax* (%) is the proportion of time N_i a given water body displayed temperatures equal or higher than *dmax*, and *t.dmax* (h) is the corresponding number of hours. *p.max.95* (%) and *t.max.95* (h) represent the same but in relation to *max.95*. *MAX.95* is the equivalent value for the weather station data.

Area /	**Open**		**Forest**			**Weather**
Water body	**Temp.pond_(Adm)**	**Perm.pond_(Ri)_1**	**Perm.pond_(Res.h)**	**Perm.pond_(Black.L)**	**Stream_(Res.h)**	**station**
N	1296 (9)	2202 (15.3)	2202 (15.3)	1008 (7)	2202 (15.3)	(32)
Mean ± SD	23.0 ± 5.1	24.3 ± 2.9	19.5 ± 1.4	20.8 ± 1.4	18.5 ± 0.9	
Min	13.0	19.5	16.2	18.4	15.8	10.0
Max	39.8	34.7	23.1	24.1	20.8	30.0
Range	26.8	15.2	6.9	5.7	5.0	20.0
CV (%)	22.4	11.9	7.0	6.7	4.8	
max.0	18.4	21.1	17.4	20.0	17.5	19.0

max.25	25.6	23.9	19.1	20.5	18.6	20.0
*dmax**	29.6	27.1	20.4	22.1	18.9	25.5
max.75	34.5	29.2	22.0	22.9	19.6	27.5
*max.95**	39.4	32.1	22.5	23.8	20.4	32.3
p.dmax (%)	11.8	16.8	25.7	18.5	31.6	
t.dmax (h)	25.5	61.7	94.5	31.0	115.8	
p.max.95 (%)	0.2	1.3	2.2	2.7	2.5	
t.max.95 (h)	0.3	4.8	8.2	4.5	9.2	
max.95 - dmax	9.9	5.0	2.1	1.6	1.5	
max.95 - MAX.95	7.2	-0.1	-9.8	-8.5	-11.8	

* Chosen summary statistics to calculate *WT*.

Table 2. Summary statistics for the CT_{max}, body mass, and *WT* (as $CT_{max} - max.95$) of species, according to analyses performed (see main text). *N* is the sample size (number of tadpoles), HR is the heating rate used in CT_{max} experiments. Superscripts at some species indicate the additional analysis(es) that a given sample was used in, besides the respective analysis where it is described.

Analysis	**Area**	**Water body**	**Family**	**Species**	***N***	**HR (°C/min)**	**CT_{max} (°C)**				
							Mean ± *SD*	*Min*	*Max*	*Range*	*CV* (%)
(1) Phylogeny vs. thermal environment	Open	Temp.pond	Hylidae	*Dendropsophus minutus*^{2,4}	18	0.1	40.9 ± 0.7	39.9	41.9	2.0	1.8
			Bufonidae	*Rhinella icterica* (1)^{2,3,4}	18	0.1	41.6 ± 0.3	40.8	41.9	1.1	0.7
	Forest	Perm.pond_(Res.h)	Hylidae	*Dendropsophus giesleri*	13	0.1	38.2 ± 0.9	35.6	38.7	3.1	2.4
				Scinax rizibilis^{2}	19	0.1	37.7 ± 0.7	36.2	38.6	2.4	1.7
		Perm.pond_(Black.L)	Hylidae	*Boana faber*	18	0.1	38.5 ± 0.4	37.8	39.2	1.4	1.1
			Odontophrynidae	*Proceratophrys boiei*^{2}	17	0.1	38.5 ± 0.2	38.0	38.8	0.8	0.6
		Stream_(Res.h)	Hylodidae	*Crossodactylus caramaschii*^{2}	20	0.1	33.0 ± 0.4	32.3	33.6	1.3	1.1

(2) Experimental HRs	Open	Temp.pond	Hylidae	*Dendropsophus minutus*	19	1	40.5 ± 0.9	39.0	41.8	2.8	2.1
			Bufonidae	*Rhinella icterica* (1)[3]	18	1	41.8 ± 0.4	41.1	42.4	1.3	0.9
	Forest	Perm.pond_(Res.h)	Hylidae	*Scinax rizibilis*	22	1	38.0 ± 0.7	35.6	38.9	3.3	1.9
	Forest	Perm.pond_(Black.L)	Odontophrynidae	*Proceratophrys boiei*	16	1	38.9 ± 0.6	37.7	39.7	2.0	1.5
	Forest	Stream_(Res.h)	Hylodidae	*Crossodactylus caramaschii*	18	1	34.5 ± 0.6	33.2	35.6	2.4	1.8
(3) Ontogeny	Open	Perm.pond_(Ri)_1	Bufonidae	*Rhinella icterica* (2)	42	0.1	40.5 ± 0.3	39.8	41.2	1.4	0.8
					31	1	40.7 ± 0.4	39.7	41.4	1.7	1.0
(4) Tadpoles vs. predator	Open	Temp.pond	Aeshnidae	*Anax concolor*	15	0.1	43.6 ± 0.6	41.6	44.3	2.7	1.5

Comparison	**Area**	**Water body**	**Family**	**Species**	***N***	**HR (°C/min)**	**Body mass (mg)**				
							Mean ± SD	*Min*	*Max*	*Range*	*CV* (%)
	Open	Temp.pond	Hylidae	*Dendropsophus minutus*[2,4]	18	0.1	412.5 ± 68.4	256.0	534.8	278.8	16.6

(1) Phylogeny vs. thermal environment	Forest		Bufonidae	*Rhinella icterica* (1)[2,3,4]	18	0.1	32.5 ± 4.3	23.5	39.1	15.6	13.2
		Perm.pond_(Res.h)	Hylidae	*Dendropsophus giesleri*	13	0.1	52.3 ± 35.6	22.5	157.3	134.8	68.0
				Scinax rizibilis[2]	19	0.1	50.9 ± 20.0	20.7	100.2	79.5	39.2
		Perm.pond_(Black.L)	Hylidae	*Boana faber*	18	0.1	509.4 ± 179.0	254.0	787.8	533.8	35.2
			Odontophrynidae	*Proceratophrys boiei*[2]	17	0.1	415.6 ± 67.3	270.9	497.5	226.6	16.2
		Stream_(Res.h)	Hylodidae	*Crossodactylus caramaschii*[2]	20	0.1	750.4 ± 300.8	115.8	1121.4	1005.6	40.1
(2) Experimental HRs	Open	Temp.pond	Hylidae	*Dendropsophus minutus*	19	1	431.2 ± 98.5	195.9	586.0	390.1	22.8
			Bufonidae	*Rhinella icterica* (1)[3]	18	1	37.4 ± 3.8	28.7	42.4	13.7	10.2
	Forest	Perm.pond_(Res.h)	Hylidae	*Scinax rizibilis*	22	1	38.6 ± 12.0	20.8	73.9	53.1	31.0
	Forest	Perm.pond_(Black.L)	Odontophrynidae	*Proceratophrys boiei*	16	1	364.9 ± 92.5	174.2	587.7	413.5	25.3

	Forest	Stream_(Res.h)	Hylodidae	*Crossodactylus caramaschii*	18	1	593.4 ± 284.2	171.6	1108.8	937.2	47.9
(3) Ontogeny	Open	Perm.pond_(Ri)_1	Bufonidae	*Rhinella icterica* (2)	42	0.1	121.6 ± 35.7	52.1	185.0	132.9	29.3
					31	1	129.1 ± 29.3	42.9	176.6	133.7	22.7
(4) Tadpoles vs. predator	Open	Temp.pond	Aeshnidae	*Anax concolor*	15	0.1	667.2 ± 191.1	297.3	1024.1	726.8	28.6

Comparison	**Area**	**Water body**	**Family**	**Species**	***N***	**HR (°C/min)**	***WT* (°C)**				
							Mean ± SD	*Min*	*Max*	*Range*	*CV* (%)
(1) Phylogeny vs. thermal environment	Open	Temp.pond	Hylidae	*Dendropsophus minutus*^{2,4}	18	0.1	1.5 ± 0.7	0.5	2.5	2.0	49.5
			Bufonidae	*Rhinella icterica* (1)^{2,3,4}	18	0.1	2.2 ± 0.3	1.4	2.5	1.1	13.2
	Forest	Perm.pond_(Res.h)	Hylidae	*Dendropsophus giesleri*	13	0.1	15.7 ± 0.9	13.1	16.2	3.1	5.8
				Scinax rizibilis^{2}	19	0.1	15.3 ± 0.7	13.7	16.1	2.4	4.3
		Perm.pond_(Black.L)	Hylidae	*Boana faber*	18	0.1	15.1 ± 0.5	14.2	15.7	1.5	3.0

			Odontophrynidae	*Proceratophrys boiei*^{2}	17	0.1	14.7 ± 0.2	14.2	15.0	0.8	1.7
		Stream_(Res.h)	Hylodidae	*Crossodactylus caramaschii*^{2}	20	0.1	12.6 ± 0.4	11.9	13.2	1.3	2.8
(2) Experimental HRs	Open	Temp.pond	Hylidae	*Dendropsophus minutus*	19	1	1.1 ± 0.9	-0.4	2.4	2.8	80.6
			Bufonidae	*Rhinella icterica* (1)^{3}	18	1	2.3 ± 0.4	1.7	3.0	1.3	15.4
	Forest	Perm.pond_(Res.h)	Hylidae	*Scinax rizibilis*	22	1	15.5 ± 0.7	13.1	16.4	3.3	4.7
	Forest	Perm.pond_(Black.L)	Odontophrynidae	*Proceratophrys boiei*	16	1	15.1 ± 0.6	13.9	15.9	2.0	3.8
	Forest	Stream_(Res.h)	Hylodidae	*Crossodactylus caramaschii*	18	1	14.1 ± 0.6	12.8	15.2	2.4	4.3
(3) Ontogeny	Open	Perm.pond_(Ri)_1	Bufonidae	*Rhinella icterica* (2)	42	0.1	8.4 ± 0.3	7.7	9.1	1.4	3.8
					31	1	8.5 ± 0.4	7.6	9.3	1.7	4.9
(4) Tadpoles vs. predator	Open	Temp.pond	Aeshnidae	*Anax concolor*	15	0.1	4.2 ± 0.6	2.2	4.9	2.7	15.4

FIGURE LEGENDS

Figure 1. (a-b) Location of the Intervales State Park (ISP) at the São Paulo state, Southeastern Brazil (QGIS 3.22.0, QGIS Association). Our study area is denoted in (b) by the small red circle at the northwest side of the ISP. (c) Satellite view of our study area (Google Earth Pro 7.3.4.8248, Google LLC ®). Sampled water bodies are inside a perimeter of 4.13 km (yellow line) and an area of ca. 1 km^2. The approximate location of the weather station of the ISP and the field laboratory are represented by the orange thermometer and the white beaker, respectively. The red line indicates 100 m linear distance.

Figure 2. Microclimatic (water temperature) and physiological (CT_{max}) diversity within our study area. Temperature distributions of HOBO data recordings from sampled water bodies, and of individual CT_{max} (both HRs) per species and samples, are presented. Probability density is proportional to the amount of data at a given temperature. In all cases, the bigger filled circle depicts the average value. Blue and red dashed lines represent *dmax* and *max.95* for each water body, respectively, whereas the black dashed line indicates the *MAX.95* of the air temperature as recorded by the ISP weather station.

Figure 3. Phylogenetic trees of our study anuran species based on TimeTree5 (Kumar et al., 2017). *Left*. Branch colors are coded according to average slow-rate CT_{max} values per species, calculated for analysis 1. *Right*. Branch colors coded according to average *WT* values (slow-rate CT_{max} – *max.95*) per species, from high (red, low *WT*) to low (blue, high *WT*) vulnerability to acute warming.

Figure 4. Sources of variation of field CT_{max} within our study anuran assemblage. (a) Phenogram displaying the evolution of tadpoles' slow-rate CT_{max} among anuran species. The topology from Figure 3 is scrambled here according to CT_{max} levels, so that tips represent present-day CT_{max} (as

measured here) and nodes are reconstructed ancestral levels. Species codes are as follows: *Ri: Rhinella icterica, Dm: Dendropsophus minutus, Pb: Proceratophrys boiei, Bf: Boana faber, Dg: Dendropsophus giesleri, Sr: Scinax rizibilis, Cc: Crossodactylus caramaschii*. Branch colors match Figure 3. (b) Phylomorphospace projection of the relationship between the average slow-rate CT_{max} of anuran species and the average of the maximum microclimatic temperatures experienced by tadpoles 24-48 h before experiments. Data points are filled according to microhabitat type and are linked by species topology. Solid error lines are 1 SD for CT_{max}, dashed error lines are 1 SD for T_{max}. An identity red line ($CT_{max} = T_{max}$) is provided. (c) Distributions of individual CT_{max} per species and HR. Big filled data points are the mean values, error bars are ± 1 SD. (d) Individual CT_{max} (both HRs) of *R. icterica* (second sample, analysis 3) along ontogeny, as per Gosner stage. Black, linked bars are predicted average CT_{max} values from the selected GAM (see main text). Size of data points is proportional to individual body mass, which is shown only for illustrative purposes.

Figure 5. Effects of using different T_{max} summary statistics (ST) on *WT* estimates. In all cases, *WT* is the difference between the slow-rate CT_{max} and a given T_{max} ST. Big filled circles are mean *WT* values. Dashed red lines at *WT* = 0 (i.e., slow-rate $CT_{max} = T_{max}$ ST) are provided for illustrative purposes. Species codes as in Figure 4.

Figure 6. Effects of sources of field CT_{max} variation on *WT* estimates. In all cases, *WT* is the difference between a specified CT_{max} and *max.95*. (a) Phenogram displaying the evolutionary patterns of *WT* (using only slow-rate CT_{max}) among anuran species. The topology is scrambled by *WT* average levels. Branch colors match Figure 3. (b) Distributions of individual *WT* per species and HR. Big filled data points are the mean values, error bars are ± 1 SD. (c) Individual *WT* (both HRs for CT_{max} data) of *R. icterica* along ontogeny, displaying data for both samples (see main text). Black, linked bars are average *WT* values predicted by a GAM analogue to that selected in analysis

3. In both (b) and (c), a dashed red line at *WT* = 0 is provided for illustration. Species codes as Figure 4.

FIGURES

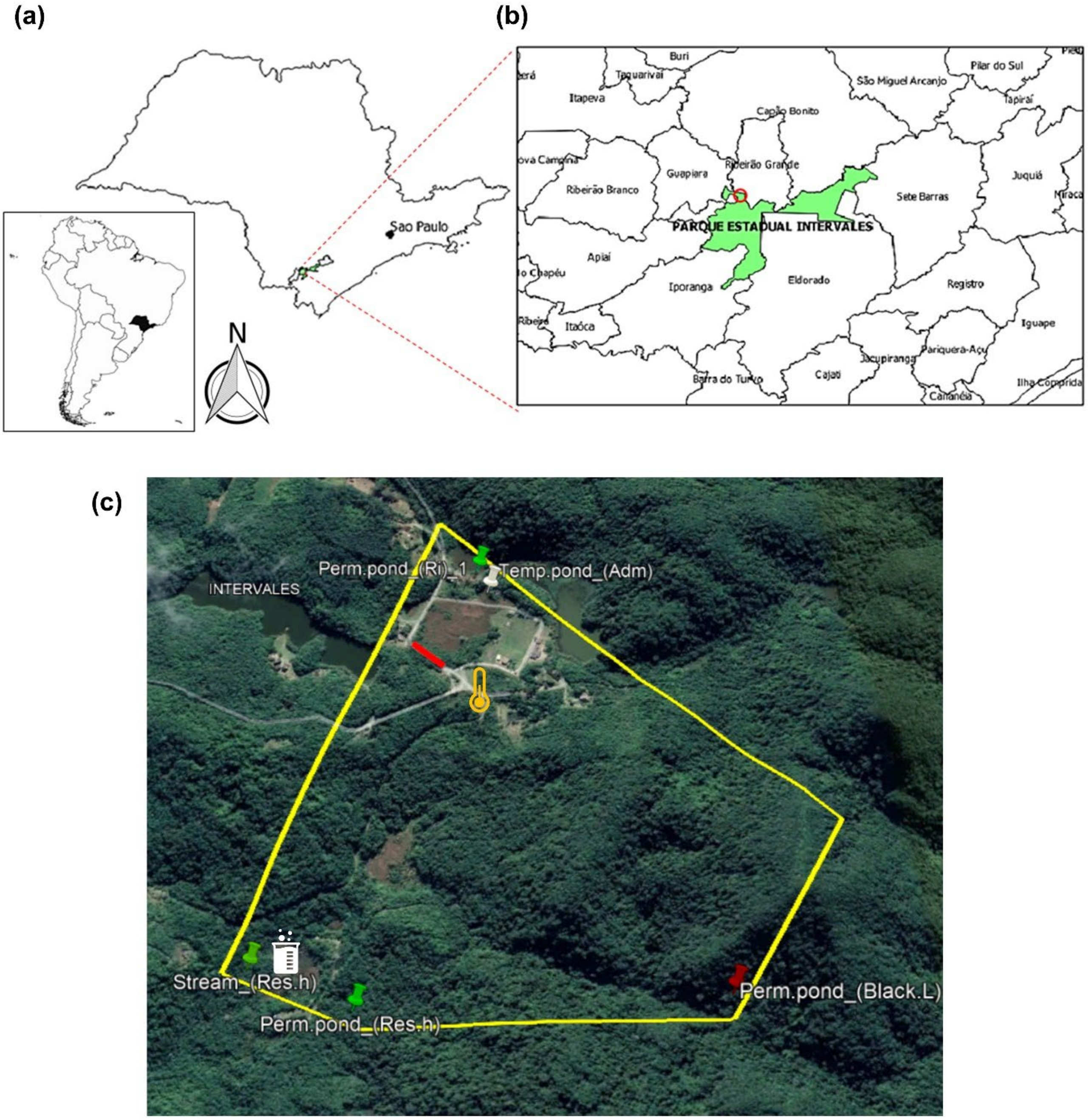

Figure 1

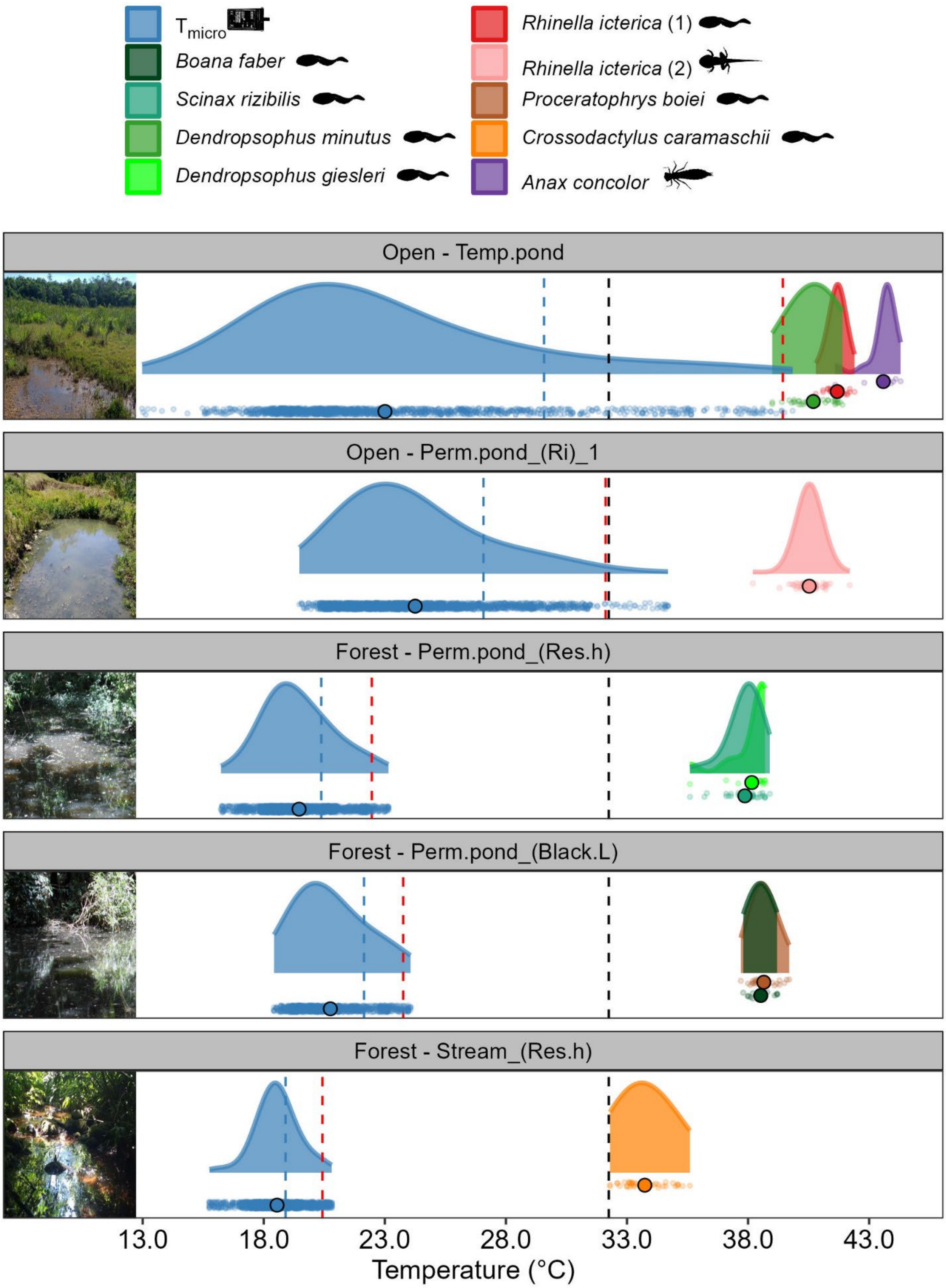
T_{micro}
Boana faber
Scinax rizibilis
Dendropsophus minutus
Dendropsophus giesleri
Rhinella icterica (1)
Rhinella icterica (2)
Proceratophrys boiei
Crossodactylus caramaschii
Anax concolor
Open - Temp.pond
Open - Perm.pond_(Ri)_1
Forest - Perm.pond_(Res.h)
Forest - Perm.pond_(Black.L)
Forest - Stream_(Res.h)
13.0
18.0
23.0
28.0
33.0
38.0
43.0
Temperature (°C)

Figure 2

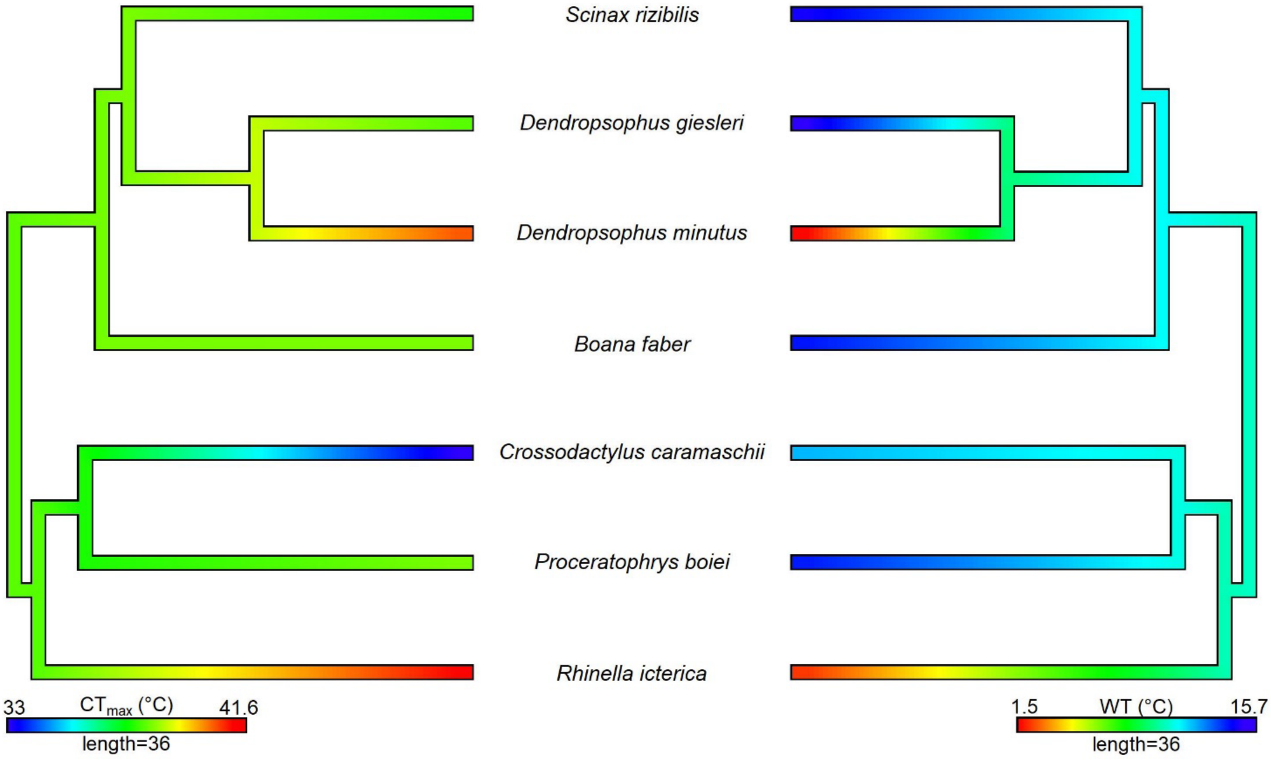
Scinax rizibilis
Dendropsophus giesleri
Dendropsophus minutus
Boana faber
Crossodactylus caramaschii
Proceratophrys boiei
Rhinella icterica
33
CT_{max} (°C)
41.6
length=36
1.5
WT (°C)
15.7
length=36

Figure 3

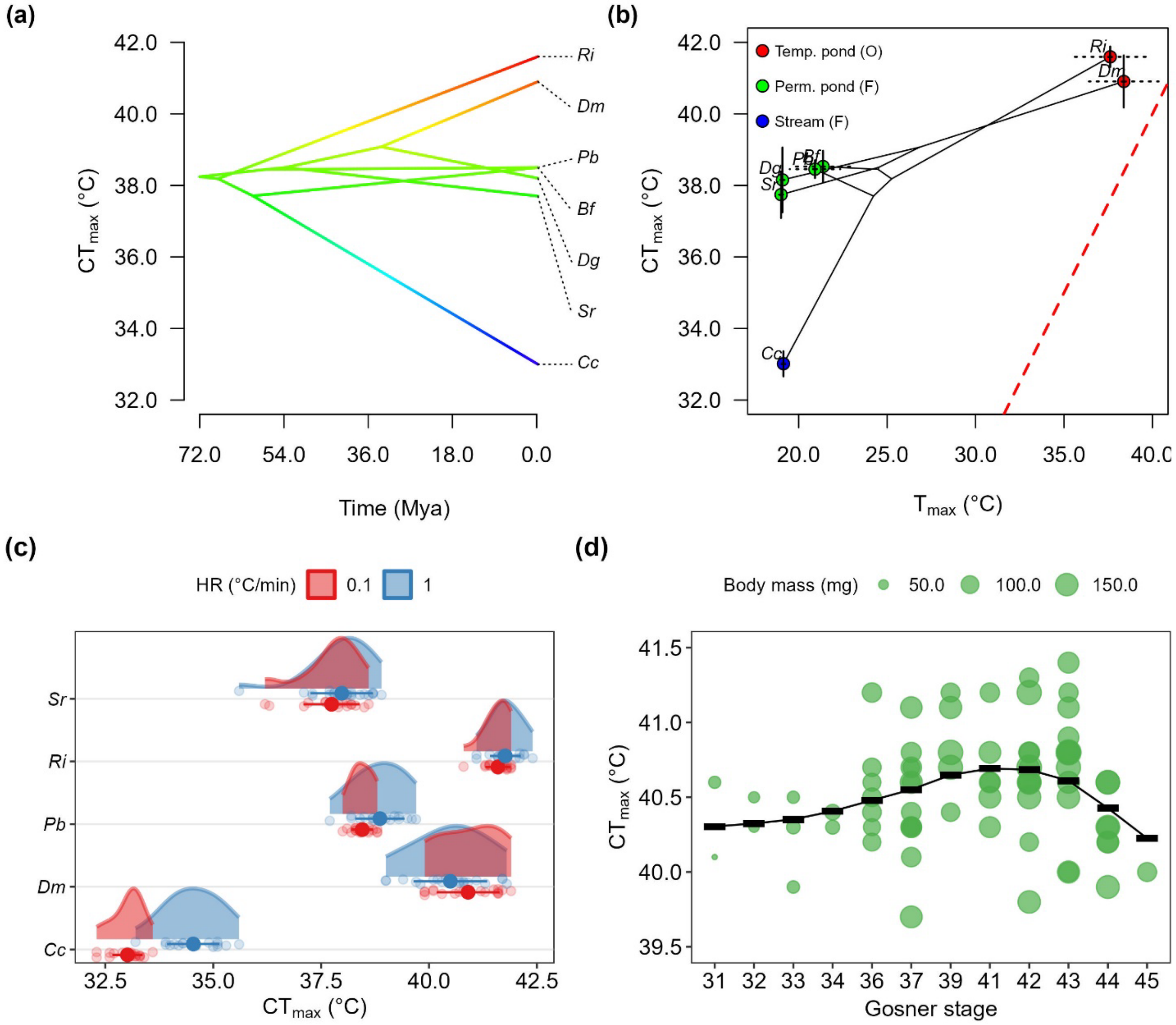
(a)
42.0
40.0
38.0
36.0
34.0
32.0
CT_{max} (°C)
72.0
54.0
36.0
18.0
0.0
Time (Mya)
Ri
Dm
Pb
Bf
Dg
Sr
Cc
(b)
Temp. pond (O)
Perm. pond (F)
Stream (F)
42.0
40.0
38.0
36.0
34.0
32.0
CT_{max} (°C)
Ri
Dm
Dg
Pb
Bf
Sr
Cc
20.0
25.0
30.0
35.0
40.
T_{max} (°C)
(c)
HR (°C/min)
0.1
1
Sr
Ri
Pb
Dm
Cc
32.5
35.0
37.5
40.0
42.5
CT_{max} (°C)
(d)
Body mass (mg)
50.0
100.0
150.0
41.5
41.0
40.5
40.0
39.5
CT_{max} (°C)
31
32
33
34
36
37
39
41
42
43
44
45
Gosner stage

Figure 4

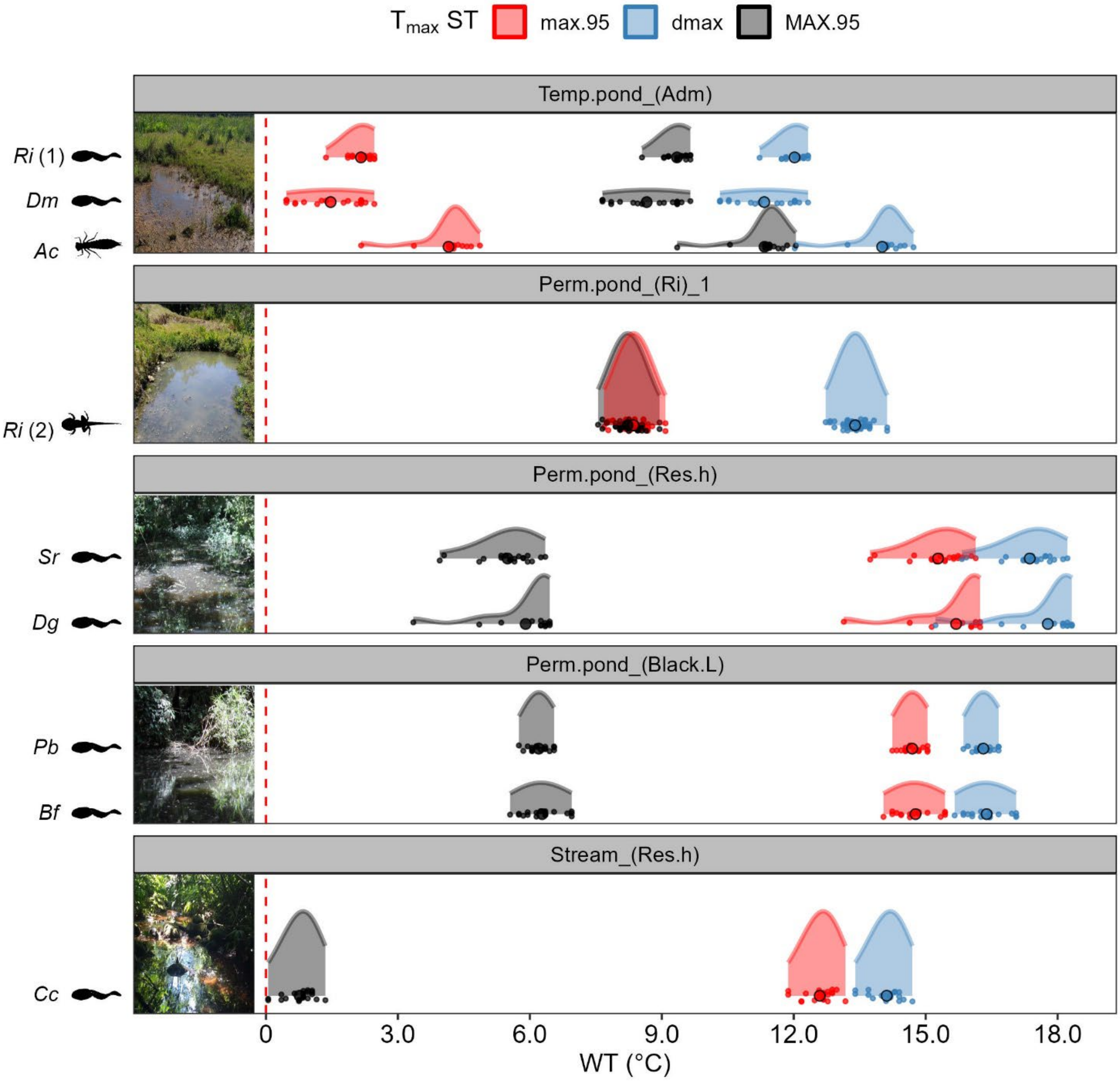
T_{max} ST
max.95
dmax
MAX.95
Temp.pond_(Adm)
Perm.pond_(Ri)_1
Perm.pond_(Res.h)
Perm.pond_(Black.L)
Stream_(Res.h)
Ri (1)
Dm
Ac
Ri (2)
Sr
Dg
Pb
Bf
Cc
0
3.0
6.0
9.0
12.0
15.0
18.0
WT (°C)

Figure 5

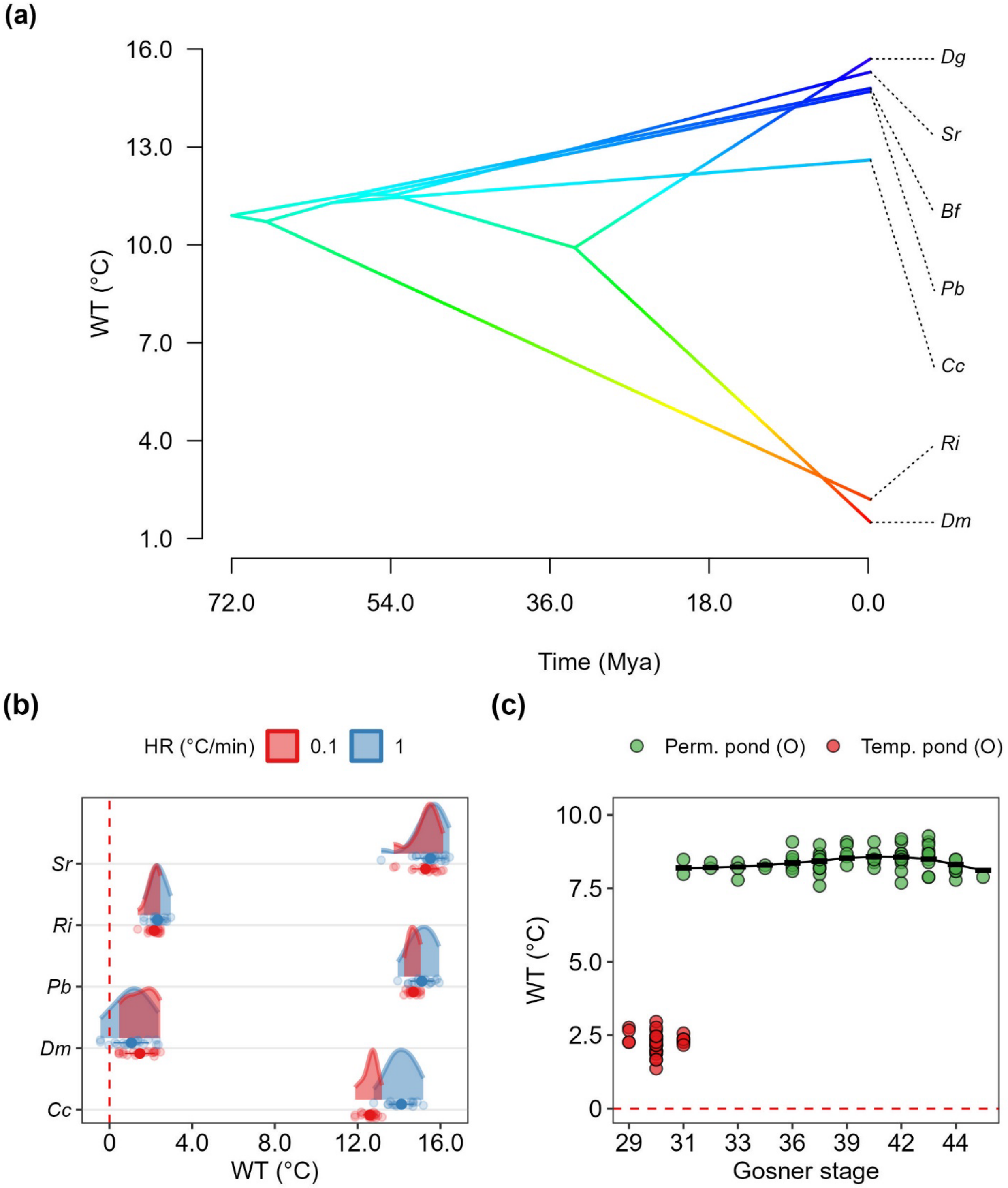
(a)
16.0
13.0
10.0
7.0
4.0
1.0
WT (°C)
72.0
54.0
36.0
18.0
0.0
Time (Mya)
Dg
Sr
Bf
Pb
Cc
Ri
Dm
(b)
HR (°C/min)
0.1
1
Sr
Ri
Pb
Dm
Cc
0
4.0
8.0
12.0
16.0
WT (°C)
(c)
Perm. pond (O)
Temp. pond (O)
10.0
7.5
5.0
2.5
0
WT (°C)
29
31
33
36
39
42
44
Gosner stage

Figure 6

Global Change Biology

SUPPLEMENTARY INFORMATION

Gustavo A. Agudelo-Cantero, Pedro L. Atencia-Gándara, Fernando R. Gomes, Carlos A. Navas.

Table S1. Competing models (hypotheses) to explain variation in CT_{max} or *WT*, according to analyses performed (see main text). Models are ranked according to their *AICc* (second-order Akaike information criterion), and the selected model is indicated in bold. *k* is the number of parameters estimated by a model; *ΔAICc* is the difference in *AICc* values between the top-ranked model and an alternative candidate model; *AICc weight* is the evidence in favor of a candidate model, among the set of models under comparison, to explain the data; and *ER* is the evidence ratio of the *AICc* weight between the top-ranked model and the second-ranked model.

Analysis / Models	*k*	*AICc*	*ΔAICc*	*AICc weight*	*ER*
(1) Phylogeny vs. thermal environment					
Testing the fit of distinct thermal environment aspects					
CT_{max} ~ Species + Body mass + T_{mean}	10	207.92	0.00	0.49	
CT_{max} ~ Species + Body mass + T_{max}	10	209.63	1.71	0.21	2.36
CT_{max} ~ Species + Body mass + T_{min}	10	210.74	2.82	0.12	
CT_{max} ~ Species + Body mass + MAX	10	212.35	4.43	0.05	
CT_{max} ~ Species + Body mass + MIN	10	212.86	4.94	0.04	
CT_{max} ~ Species + Body mass + dT	10	213.87	5.94	0.03	
CT_{max} ~ Species + Body mass + dCV	10	214.13	6.21	0.02	
CT_{max} ~ Species + Body mass + HR_{eco}	10	214.23	6.31	0.02	
CT_{max} ~ Species + Body mass + DT	10	214.45	6.53	0.02	
Testing the fit of distinct thermal environment aspects					
CT_{max} ~ Species + Body mass + T_{max}	9	207.27	0.00	0.59	
CT_{max} ~ Species + T_{max}	10	209.63	2.36	0.18	3.26
CT_{max} ~ Species + Body mass	8	209.75	2.48	0.17	
CT_{max} ~ Species	9	212.08	4.81	0.05	

CT_{max} ~ 1	2	600.38	393.11	0.00	
(2) Experimental HRs					
CT_{max} ~ Species* × *HR	11	336.61	0.00	1.00	
CT_{max} ~ Species + HR	7	378.97	42.36	0.00	
CT_{max} ~ Species	6	392.76	56.15	0.00	
(3) Ontogeny					
***CT_{max} ~ HR* + s(*Gosner stage*, bs = "ordinal", m = 2, by = *HR*)**	7.33	59.85	0.00	0.60	
CT_{max} ~ s(*Gosner stage*, bs = "ordinal", m = 2)	6.4	60.90	1.05	0.36	1.67
CT_{max} ~ 1	2	65.32	5.48	0.04	
(4) Tadpoles vs. predator					
CT_{max} ~ Species, variable variances among species	6	85.57	0.00	0.99	
CT_{max} ~ Species, equal variances among species	4	94.79	9.22	0.01	100.36
CT_{max} ~ 1	2	170.77	85.20	0.00	
Species differences in *WT*					
WT ~ Species, variable variances among species	18	236.82	0.00	1.00	
WT ~ Species, equal variances among species	20	279.38	42.55	0.00	

Table S2. Papers published in *Global Change Biology* (1995 - 2022), as per a search at the journal website on 24 November 2022 applying the following criteria: *"interindividual variation" OR "inter-individual variation" OR "interindividual variability" OR "inter-individual variability" OR "interindividual variance" OR "inter-individual variance" OR "among-individual variation" OR "among-individual variability" OR "among-individual variance" OR "variation among individuals" OR "differences among individuals" OR "variability among individuals"*. Type of publication, according to the journal's categories, is identified. The "Context" column indicates whether interindividual variation was (1) only mentioned in the paper, (2) controlled (e.g., in a mixed-effect modeling framework) to focus on estimation of mean effects, or (3) actually included in the investigation with a particular focus.

No.	Reference (by publication date)	Type	Context
1	Maxwell, T. L., Canarini, A., Bogdanovic, I., Böckle, T., Martin, V., Noll, L., Prommer, J., Séneca, J., Simon, E., Piepho, H., Herndl, M., Pötsch, E. M., Kaiser, C., Richter, A., Bahn, M., & Wanek, W. (2022). Contrasting drivers of belowground nitrogen cycling in a montane grassland exposed to a multifactorial global change experiment with elevated CO_2, warming, and drought. *Global Change Biology*, *28*(7), 2425–2441. https://doi.org/10.1111/gcb.16035	Research Article	Controlled
2	Wu, Q., Miles, D. B., Richard, M., Rutschmann, A., & Clobert, J. (2022). Intraspecific diversity alters the relationship between climate change and parasitism in a polymorphic ectotherm. *Global Change Biology*, *28*(4), 1301–1314. https://doi.org/https://doi.org/10.1111/gcb.16018	Research Article	Controlled
3	Marchin, R. M., Backes, D., Ossola, A., Leishman, M. R., Tjoelker, M. G., & Ellsworth, D. S. (2022). Extreme heat increases stomatal conductance and drought-induced mortality risk in vulnerable plant species. *Global Change Biology*, *28*(3), 1133–1146. https://doi.org/https://doi.org/10.1111/gcb.15976	Research Article	Controlled
4	Fellous, A., Wegner, K. M., John, U., Mark, F. C., & Shama, L. N. S. (2022). Windows of opportunity: Ocean warming shapes temperature-sensitive epigenetic reprogramming and gene expression across gametogenesis and embryogenesis in marine stickleback. *Global Change Biology*, *28*(1), 54–71. https://doi.org/https://doi.org/10.1111/gcb.15942	Research Article	Mentioned

5	Anderson-Teixeira, K. J., Herrmann, V., Rollinson, C. R., Gonzalez, B., Gonzalez-Akre, E. B., Pederson, N., Alexander, M. R., Allen, C. D., Alfaro-Sánchez, R., Awada, T., Baltzer, J. L., Baker, P. J., Birch, J. D., Bunyavejchewin, S., Cherubini, P., Davies, S. J., Dow, C., Helcoski, R., Kašpar, J., … Zuidema, P. A. (2022). Joint effects of climate, tree size, and year on annual tree growth derived from tree-ring records of ten globally distributed forests. *Global Change Biology*, *28*(1), 245–266. https://doi.org/https://doi.org/10.1111/gcb.15934	Research Article	Controlled
6	Bairos-Novak, K. R., Hoogenboom, M. O., van Oppen, M. J. H., & Connolly, S. R. (2021). Coral adaptation to climate change: Meta-analysis reveals high heritability across multiple traits. *Global Change Biology*, *27*(22), 5694–5710. https://doi.org/https://doi.org/10.1111/gcb.15829	Research Article	Mentioned
7	Liao, M., Li, G., Wang, J., Marshall, D. J., Hui, T. Y., Ma, S., Zhang, Y., Helmuth, B., & Dong, Y. (2021). Physiological determinants of biogeography: The importance of metabolic depression to heat tolerance. *Global Change Biology*, *27*(11), 2561–2579. https://doi.org/10.1111/gcb.15578	Research Article	Investigated
8	Kershaw, J. L., Ramp, C. A., Sears, R., Plourde, S., Brosset, P., Miller, P. J. O., & Hall, A. J. (2021). Declining reproductive success in the Gulf of St. Lawrence's humpback whales (Megaptera novaeangliae) reflects ecosystem shifts on their feeding grounds. *Global Change Biology*, *27*(5), 1027–1041. https://doi.org/https://doi.org/10.1111/gcb.15466	Research Article	Controlled
9	Telesca, L., Peck, L. S., Backeljau, T., Heinig, M. F., & Harper, E. M. (2021). A century of coping with environmental and ecological changes via compensatory biomineralization in mussels. *Global Change Biology*, *27*(3), 624–639. https://doi.org/https://doi.org/10.1111/gcb.15417	Research Article	Controlled
10	Bideault, A., Galiana, N., Zelnik, Y. R., Gravel, D., Loreau, M., Barbier, M., & Sentis, A. (2021). Thermal mismatches in biological rates determine trophic control and biomass distribution under warming. *Global Change Biology*, *27*(2), 257–269. https://doi.org/10.1111/gcb.15395	Research Article	Mentioned
11	Burraco, P., Orizaola, G., Monaghan, P., & Metcalfe, N. B. (2020). Climate change and ageing in ectotherms. *Global Change Biology*, *26*(10), 5371–5381. https://doi.org/https://doi.org/10.1111/gcb.15305	Opinion	Mentioned
12	Denechaud, C., Smoliński, S., Geffen, A. J., Godiksen, J. A., & Campana, S. E. (2020). A century of fish growth in relation to climate change, population dynamics and exploitation. *Global Change Biology*, *26*(10), 5661–5678. https://doi.org/10.1111/gcb.15298	Research Article	Investigated

13	Caldwell, T. J., Chandra, S., Feher, K., Simmons, J. B., & Hogan, Z. (2020). Ecosystem response to earlier ice break-up date: Climate-driven changes to water temperature, lake-habitat-specific production, and trout habitat and resource use. *Global Change Biology*, *26*(10), 5475–5491. https://doi.org/https://doi.org/10.1111/gcb.15258	Research Article	Mentioned
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Figure S1. *CT*$_{max}$ diversity within the anuran assemblage. Three *CT*$_{max}$ distributions are observed discretely separated and with distinct *CT*$_{max}$ modes (excess mass test, statistic = 0.14, $P < 0.01$; (Ameijeiras-Alonso et al., 2019), peaking at 33.1, 38.4, and 41.6°C (dashed black lines). *CT*$_{max}$ groups are bounded by minimum and maximum values within broad microhabitat categories, as coded by colors.

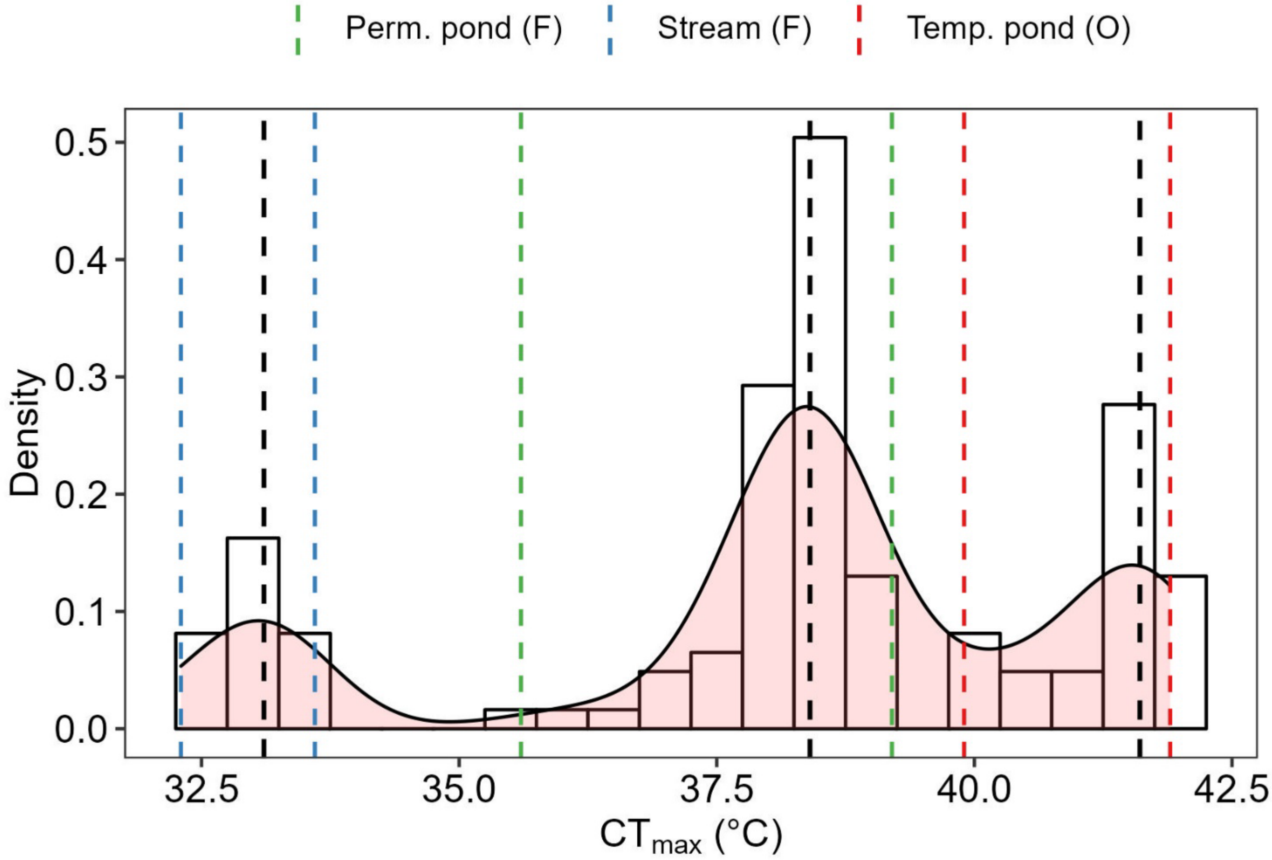


Figure S2. Relationships between effects of experimental heating rates (HRs, 1 or 0.1°C/min) on average vs. interindividual variation of CT_{max} among anuran species. In both cases, the *x*-axis is the difference (in °C) between the average fast-rate CT_{max} and the average slow-rate CT_{max}. In (a), the *y*-axis is the proportional reduction of the standard deviation of CT_{max} at the slow HR relative to the fast HR. The same is shown in (b) for the range of individual CT_{max}.

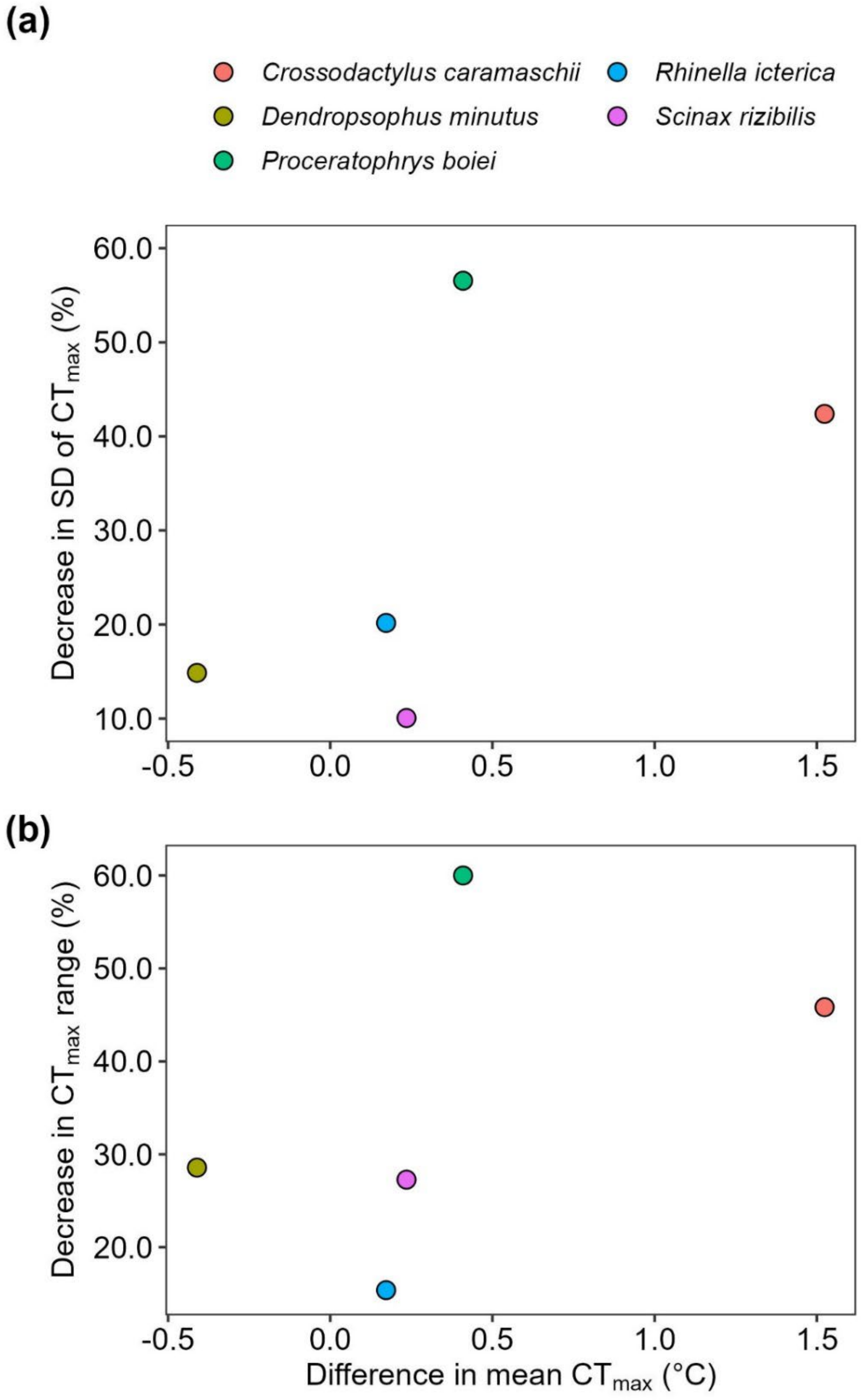
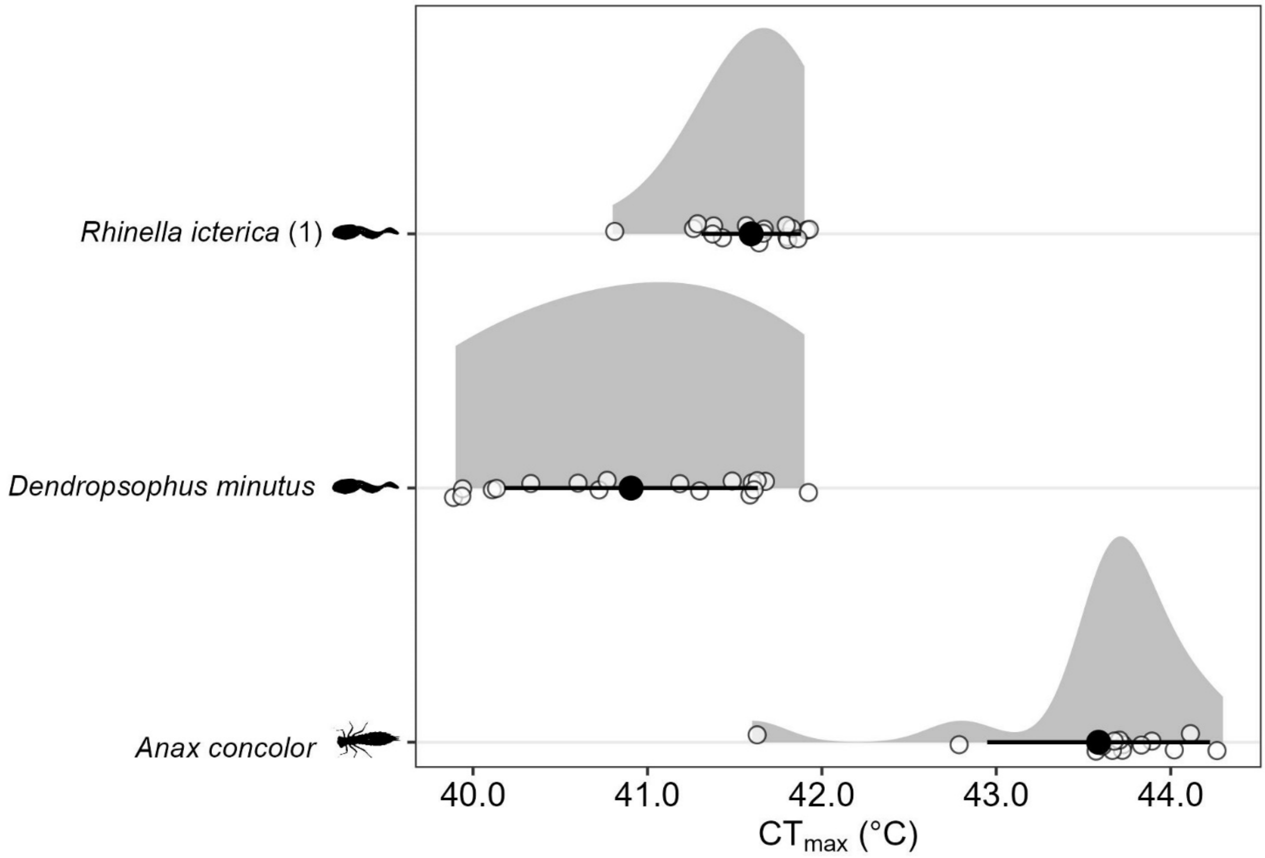


Figure S3. Field *CT*$_{max}$ variation between anuran species and a syntopic predator, cohabiting the same open temporary pond. Both individual (white dots) and average (black dots) *CT*$_{max}$ values are presented, as well as ± 1 SD (error bars).



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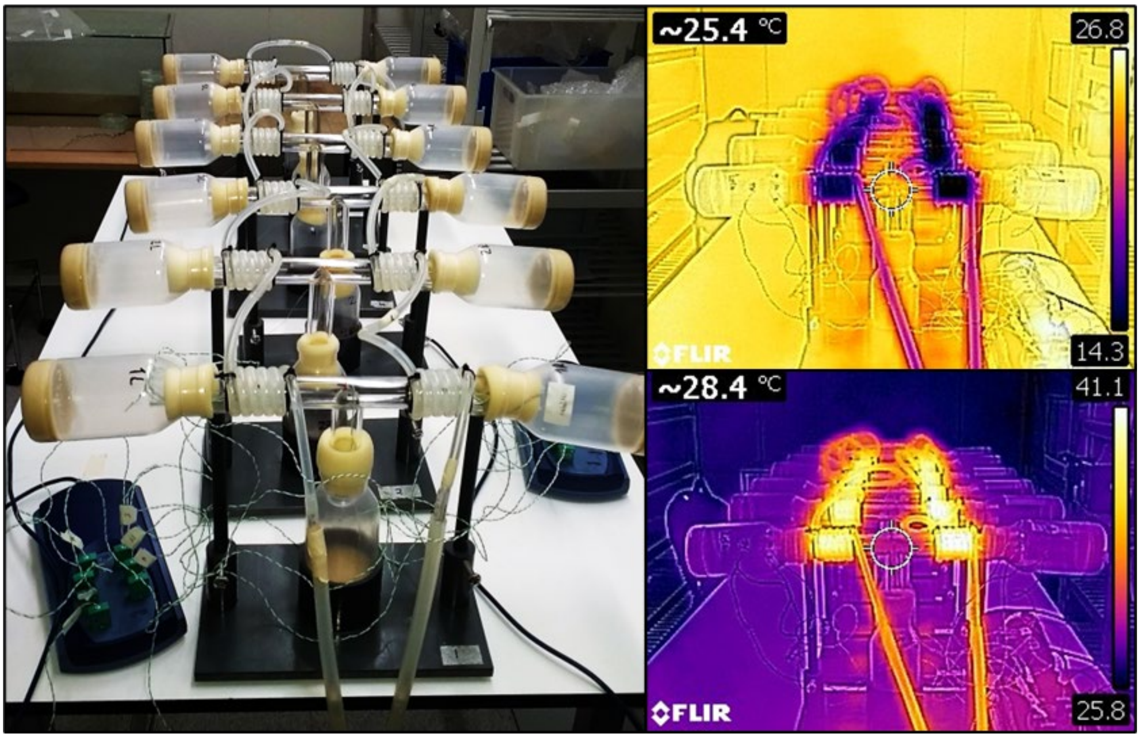
Chapter 3:

“Navigation behaviors of fruit flies in spatially explicit thermal landscapes”



Module of Thermal Decision Systems and experimental thermal landscapes used to study navigation behaviors of fruit flies

Nature Ecology & Evolution

Navigation behaviors of fruit flies in spatially explicit thermal landscapes

Gustavo A. Agudelo-Cantero[1,2,*], Jesper Givskov Sørensen[2], Palle Duun Rohde[3], Carlos A. Navas[1†], Volker Loeschcke[2†].

[1] Department of Physiology, Institute of Biosciences, University of São Paulo, Rua do Matão 101, Tv 14, 05508-090 São Paulo, Brazil.

[2] Department of Biology - Genetics, Ecology and Evolution, Faculty of Natural Sciences, Aarhus University. Ny Munkegade 116, 8000 Aarhus, Denmark.

[3] Department of Chemistry and Bioscience, Faculty of Engineering and Science, Aalborg University. Frederik Bajers Vej 7H, 9220 Aalborg, Denmark.

[*] Corresponding author: gustavo.agudelo@ib.usp.br, gustavo.a.agudelo@gmail.com

[†]CAN and VL are both senior authors.

Running title: Navigation behaviors in thermal landscapes

Abstract

Motile animals use behavior to avoid thermal extremes in their environments, but alternative behaviors to thermophobia may coexist within populations. Hereby we used the newly-introduced module of Thermal Decision Systems (TDSs) to study the navigation behaviors of fruit flies (*Drosophila melanogaster*) in an extremely cold or hot experimental thermal landscapes (CTL or HTL). We used an isofemale line approach to estimate total genetic variance, broad-sense isofemale heritability, and evolutionary potential of navigation behaviors. Fruit flies displayed diverse behaviors in the TDSs, from the exploration or avoidance of the thermal landscape, to the voluntary tendency to engage in thermal risks or avoid them (thermal boldness or thermal shyness). The proportion of flies in a sample displaying a particular behavior varied among isofemale lines, deriving on behavioral diversity at the population level. Both exploration and thermal boldness were higher in the HTL relative to the CTL. Furthermore, fly behaviors displayed higher (total) genetic variance among isofemale lines, broad-sense heritability, and evolvability in the HTL than in the CTL, and behaviors were not genetically correlated between thermal landscapes. Our results indicate that the configuration of the thermal landscape modulate navigation behaviors of fruit flies, including their evolutionary potential.

Keywords: Thermal boldness, volunteer exploration, extreme temperatures, ectothermic animals, isofemale lines, heritability.

Introduction

With few exceptions (e.g., abyssal zones, caves and other subterranean habitats), most terrestrial and aquatic environments are thermally variable in both time and space [1–3]. Consequently, organisms rarely experience average thermal conditions, but are rather exposed to different forms of thermal variations in their microclimates [4–6]. In time, individual organisms experience both "normal" thermal fluctuations (i.e., conditions common across generations) and rare extreme events (within and across generations, e.g., heatwaves) [7–9]. Across space, microclimates are frequently thermally heterogeneous [10–12]. In response, animals integrate physiological and behavioral strategies to regulate their body temperature (T_b) within a range that potentially maximizes fitness [13–15]. Yet, the contribution of physiology or behavior to thermoregulation varies with ontogeny (e.g., embryos vs. larvae/juveniles vs. adults), dispersal capabilities (e.g., sessile vs. motile forms) and environmental opportunities (e.g., thermally constant vs. variable microclimates) [2,8,16]. Thus, and at the short-term (seconds to hours), the first and immediate line of response of motile individuals to a spatiotemporally dynamic thermal environment (hereafter thermal landscape) is primarily behavioral [2,3,17].

Behavioral strategies to thermoregulate are particularly relevant and diverse in ectothermic animals, which are more constrained in their capacity for endogenous heat production relative to endothermic counterparts [18,19]. Changes in T_b affect behavior of ectothermic animals through bottom-up kinetic effects (via thermodynamic constraints on biological rates over a range of time scales) and top-down integrated effects (via perception and integration of thermal information) [20]. Bottom-up effects have been traditionally studied from an optimality perspective typified by thermal performance curves [21], including the flexibility (plastic or evolutionary) of these curves (or some specific parameters) [18,22]. On the other hand, top-down effects have been investigated primarily regarding aspects of animal navigation and orientation [23–25]. Furthermore, changes in behavior (namely behavioral thermoregulation) have the potential to modify individual T_b [20,26,27]. In this context, body size plays an important role. Whereas medium- (0.1 to 1 kg) and large-bodied (>

1 kg) individuals integrate slowly microclimatic temperature (T_e) into T_b while moving across a thermal landscape, the T_b of small-bodied individuals (< 0.1 kg) track faster T_e given their little thermal inertia [3,28,29]. At the same time, small-bodied individuals would have access to much diverse thermal landscapes within short distances [3,11]. Behavioral aspects of thermal biology are well known in some lineages of medium-bodied ectothermic animals (e.g., heliothermic lizards) [30,31]. However, less is known for small-bodied counterparts (e.g., small insects of less than 10 mg [29]) in this regard relative to aspects of their thermal physiology [32–34].

From an optimality perspective [18,21], motile ectothermic animals should avoid extreme low and high temperatures that might compromise their performance or even kill them, respectively [35]. In fact, avoidance of extreme temperatures is a widespread response among motile ectothermic animals and perhaps the most ancestral type of thermoregulatory behavior [36]. Furthermore, avoidance of high temperatures is considered essential for those ectothermic animals living close to their upper thermal limits [37], given that plastic and evolutionary responses of heat tolerance might be insufficient to keep up with current and future rates of habitat warming [38–40]. Such scenario is even more critical for small ectothermic animals like many insects [3,37,41], given constraints posed by a small body size (see above). However, small motile insects may also exhibit alternative behaviors when navigating in extreme thermal landscapes (ETLs), including the volunteer exploration of extreme temperatures and thermal risk-taking (namely thermal boldness) [42,43]. Moreover, thermal boldness towards extreme high temperatures may bear little dialog with acute heat tolerance, thus departing from any expectation based on optimality models of thermal adaptation (see Fig. 8 in [42]). Hence, navigation of small motile ectothermic animals in ETLs should involve complex cognitive, decision-making processes through top-down thermal effects [20,44], resulting in a variety of behaviors modulating individual exposure to thermal extremes [42]. Yet, thermal decision rules, perhaps the most basic aspect of animal navigation in ETLs [44], remain understudied in small motile ectothermic animals.

Recently, Navas et al. (2022) introduced the *Thermal Decision Systems* (TDSs), a module of replicated T-shaped labyrinths (hereafter T-System) to study navigation behaviors of small insects in spatially explicit thermal landscapes [42]. Unlike typical linear thermal gradients employed to study thermal selection, animal movement beyond areas of extreme temperatures (Critical Temperature Zones, CTZs) at both ends of a TDS replicate is possible and, in fact, necessary for individuals (usually starved) to access food. Using the TDSs, the researchers observed that a fraction of fruit flies (*Drosophila melanogaster*) in an observational group (fly sample) were thermally bold and crossed through either a cold-CTZ or hot-CTZ [42]. Yet, fly behavior in a TDS is complex and guided by, at least, two navigation decisions (Fig. 1A). The **first decision** is faced by all individuals in the fly sample and relates to their *reaction towards novelty*: staying in the known and thermally constant initial container (*avoidance*), or move towards the novel and thermally dynamic T-System (*exploration*). The **second decision** is faced only by explorer individuals and relates to their *inclination to engage (or not) in a thermal risk*: entering and crossing a CTZ towards a lateral container (*thermal boldness*), or keep exploring the T-System without crossing any CTZ (*thermal shyness*). We borrowed terminology from animal temperament research [45] for operational purposes without making any *a priori* assumptions about animal personality, an endeavor that would require validation. Thus, our behavioral categories mean to guide hypothesis testing under a formal framework that integrates thermal biology and animal personality [45,46], without neglecting the multidimensional nature of animal behavior [47].

Based on their observations, Navas et al. (2022) postulated two hypotheses regarding the population-level (1) phenotypic variation in navigation behaviors of fruit flies in ETLs, and (2) the genetic basis and heritability underlying such variation. The first hypothesis states that individuals exploring an extreme thermal landscape would distribute along a bold-shy continuum, and population patterns would be context-specific. Although this hypothesis admits a number of predictions (see Fig. 7 in [42]), we illustrate the extreme cases. From an optimality perspective, *behavior is conservation,* and most explorer individuals would exhibit thermal shyness, though a small fraction would be thermally bold. At the other end, *behavior is opportunity*, and so most explorer individuals would be thermally bold. The second hypothesis posits that navigation behaviors of small motile ectothermic animals in ETLs are genetically variable within a population, and such genetic variation is heritable. This hypothesis is based on observed differences in thermal risk-taking behaviors among fly lineages with distinct genetic makeup [42]. This paper tackles both hypotheses in fruit flies (*D. melanogaster*).

Fruit flies constitute an excellent model to study the behavior of small motile ectothermic animals in ETLs for a number of reasons. Much is known about the physiological and molecular underpinnings of thermal adaptation in fruit flies [48–50], including aspects of their thermal sensation systems [51–53]. Yet, thermal behavior, an important component of their natural history [54], has been comparatively less studied [55]. Furthermore, fruit flies have impressive dispersal capabilities and can travel over 15 km (ca. 6 million body sizes!) through the desert in a single night [56,57]. Then, behavioral decisions regarding their thermal environment should be an important component of their navigation and orientation. In addition, fruit flies display individuality in distinct behaviors [58–60], including thermotaxis [47]. These aspects, combined with the possibility to use isogenic lines of flies [61], create an ideal system to investigate the population-level variation and heritability of navigation behaviors of small motile ectothermic animals in ETLs. Therefore, by taking advantage of the TDSs, we set two different ETLs and studied behavioral responses of 25 highly inbred isofemale lines (ILs) of *D. melanogaster* in extreme cold or heat. We further asked, without any *a priori* hypothesis, whether fruit flies display sex differences in their navigation behaviors in ETLs. Furthermore, we explored the context dependency of navigation behaviors of fruit flies by assessing their correlation (phenotypically and genotypically) between ETLs.

Material and methods

Experimental system and thermal landscapes

We modified the original TDS design to set up two temporally constant, yet spatially heterogeneous experimental thermal landscapes (Fig. S1). For a detailed description of the system and its parts, we refer readers to the original publication. Briefly, we connected six TDSs to a single temperature-controlled water bath (NESLAB, RTE-300D). Water circulated the system from the first TDS (T1) through the last (T6), turned back in the opposite order (T6 through to T1), and returned to the bath. This setup aimed to establish similar temperatures in the middle spots of CTZs withing (left and right sides) and between TDSs. Thus, we set the thermal bath at 12°C for creating a Cold-CTZs Thermal Landscape (CTL; Fig. S1A), with a Cold-Warm-Cold thermal gradient in the horizontal portion of the T-System. Alternatively, we set the thermal bath at 42°C for creating a Hot-CTZs Thermal Landscape (HTL; Fig. S1B), with a Hot-Warm-Hot thermal gradient. We determined such set-point temperatures in a set of pilot tests to target specific conditions limiting fly transit through CTZs (Appendix 1; see below). Our single-bath setup proved thermally stable in the time course of the experiments, with maximum loss in cooling/heating power involving a temperature difference of < 1°C between the right (water entry from the bath) and left (water exit to the bath) CTZs of T1 (Appendix 1).

During pilot tests, we monitored temperatures in key internal spots of T1 by placing type K thermocouples and recorded air temperatures via TC-08 data loggers (Pico Technology, UK) at 1 s intervals. Within T1, the temperature difference between CTZs is higher than in any other TDS, so temperature measurements in T1 are a conservative representation of thermal conditions of TDSs. We continued registering temperatures of the system in formal experiments (CTL: $N = 30$; HTL: $N = 29$) and characterized average conditions of each thermal landscape, which are depicted in lower panels of Figure S1. We maintained the room temperature at 26 ± 1°C throughout experiments, so that temperatures in home bottles, feeding bottles, and ascending tubes followed closely the room temperature. In the middle of the thermal gradient, temperatures were close to 25°C in CTL and 28°C in HTL experiments, respectively. Temperature decreased (CTL) or increased (HTL) by ca. 9°C in only 3.5 cm, the distance between the middle of the thermal gradient and any inner black ring marking the onset of CTZs, on both sides of the T-System. The middle spot of CTZs, where temperatures of the system were most extreme, were around 13.3 (right CTZ) – 14.1°C (left CTZ) in CTL experiments, and 40.6 (right CTZ) – 40.1°C (left) in HTL experiments. These temperatures corresponded to the *maximum cold* and *minimum hot* temperatures at which crosses occurred for an exploratory set of five IL during pilot tests (Appendix 1). From the middle of CTZs to the outer black rings, temperature increased by ca. 4°C in CTL or decreased up to 5°C in HTL experiments, respectively.

Flies maintenance and handling

To obtain fly samples that differed in their genetic background, we used 25 IL of *D. melanogaster* randomly selected from a stock of 33, previously established from an Australian population and provided by Prof. J.S.F. Barker (University of New England, Australia). This IL stock was kept in shell 7mL vials and standard *Drosophila* oatmeal–sugar–yeast–agar medium since their arrival at the Department of Biology - Genetics, Ecology and Evolution, Aarhus University (January 2020). Room temperature and photoperiod were set at 19 ± 1°C and 12L:12D (fly photophase from 0800-2000 CET), respectively. To maintain the IL stock, we tipped each IL four times every three days per generation (ca. 17-19 days), discarding the poor performing tip 1 and using tips 2-4 to set the new generation every time.

Since we had five TDSs (T2-T6) available for observation, we worked with sets of 5 IL randomly selected in each experimental round (CTL or HTL). Then, from each IL we obtained one fly sample per sex according to the following procedure. We selected ca. 50 pairs per IL from stock vials and transferred them to a 8 oz. (ca. 236.6 mL) fresh food bottle to lay eggs for three days. We tipped the bottle thrice every three days to produce parental flies. When parental flies were 3-4 days old, we selected about 40 pairs/spoon to lay eggs overnight (three spoons, accounting for egg viability). In the next morning, we collected ca. 60 ± 5 eggs from each spoon and placed them separately into fresh 7 mL food vials. Thus, fly development was density-controlled, and occurred under the same temperature and photoperiod conditions established for the IL stock.

First flies emerged in the afternoon of day 17 after setting egg laying and were discarded. On day 18, we collected virgin flies during early (0000-0200) and mid (0700-0800) fly photophase. Since egg and developmental viability was rather low, we pooled the set of three developmental vials to ensure adequate numbers of virgin flies of both sexes for experimentation. Then, we sorted and counted flies by sex under CO_2 anesthesia, and transferred females and males separately into fresh 7 mL food vials to recover. We repeated this procedure in the morning of the next day (0000-0200 into fly photophase) and focused particularly on collecting males, since most flies emerging on day 18 were females. Three days after (day 21), virgin females and males were transferred to fresh-food vials and re-counted (by discounting dead flies). Thus, we obtained two fly samples applying this protocol, each composed by virgin males or females (15-64 individuals) of the same IL identity that, although shared the same developmental environment, were tested separately.

The entire procedure described above was triplicated per isofemale line and experiment (CTL or HTL) in order to test each line-sex independently three times in each thermal landscape. Thus, our ideal experimental design was: 3 fly samples × 2 sexes × 25 isofemale lines × 2 thermal landscapes = 75 fly samples/sex to be tested in the CTL, and 75 fly/sex to be tested in the HTL. However, isofemale lines differed in egg-to-adult viability and mortality, so for some lines-sex we only could obtain and test two fly samples. Consequently, our effective design was: 1) In the CTL: 65 fly samples – females, 61 fly samples – males; and 2) in the HTL: 69 fly samples – females, 60 fly samples – males.

Fly behavior

To test fly behavior in both sexes separately on the same day, we randomized the order of experiments according to sex (i.e., females or males first). Yet, we used the same thermal landscape configuration (CTL or HTL) within an experimental day for both sexes. The first experiment was always conducted between 0100-0200 into fly photophase, and the second experiment was always performed between 0230-0330 into fly photophase. On day 24 (after setting egg laying), we transported fly samples 18 h before the respective experiment from the developmental room at 19 ± 1°C to the experimental room at 26 ± 1°C. Then, we transferred flies to home bottles (but not yet installed in TDSs) with agar and no food for starvation previous to experiments, and counted flies again. In some cases, few flies died during starvation time. Then, we counted flies alive on the experimental day in all fly samples, and consider these counts as the number of experimental flies in samples. Flies were 7 days old in most cases with few exceptions (range 5-8 days old). For more details on the experimental protocol, we refer readers to Appendix 1.

We turned the thermal bath on 40 min before starting the first experiment to ensure the system was thermally stable. Next, we randomly assigned fly samples (then, ILs) among operative TDSs and maintained IL position for the second experiment with the other sex. Afterwards, we installed home bottles in TDSs 15 min before experiments. At time 0' (t_0) the experiment started by removing the stopper from the ascending tube, which granted fly access to the T-System. Fly behavior was monitored by one observer for 1 h (duration of experiments), but every 10 min both the number of flies in the T-System and the number of flies in feeding bottles (i.e., after crossing CTZs) were counted. After training in pilot tests, the observer took about 10 sec/TDS to count flies, so all TDSs were counted (from T2-T6) in ca. 1 min. From these counts, and knowing the number of flies in each sample, we estimated: 1) the number of **explorers**, flies that left home bottles and were either in the T-System or in feeding bottles (the sum of both); 2) the number of **avoiders**, flies that stayed in home bottles (*total* - *explorers*); 3) the number of **bold** explorers, flies that crossed CTZs and reached feeding bottles; and 4) the number of **shy** explorers, flies that stayed in the T-System and did not cross through a CTZ.

Given that fly samples had different sizes, we operationalized fly behaviors as follows: 1) **proportion of fly sample ($\boldsymbol{Y_1}$)**, i.e., the proportion of all flies in a fly sample that exhibited a given navigation behavior; and 2) **proportion of explorer flies ($\boldsymbol{Y_2}$)**, i.e., the proportion of flies out of those exploring the system that exhibited either thermal boldness or shyness. While Y_1 informs the occurrence of navigation behaviors in the population of isofemale lines tested, Y_2 analyzes thermal boldness and shyness as behavioral choices derived from an exploratory driver.

Data analyses

All data analyses and visualization were performed in the R programming environment (version 4.1.2) [62]. We performed extensive data exploration previous to formal analyses, which are thoroughly described in our Supplementary R Script (Appendix 2). We refer interested readers to this file for further details on our data analysis approach.

1. *Temporal dynamics of fly navigation behaviors*

To describe the temporal dynamics of fly navigation behaviors in each thermal landscape, according to a given navigation decision (1st: avoidance vs. exploration, 2nd: boldness vs. shyness), we fitted loess curves to the data. We assessed whether fly navigation changed over time by identifying inflection points on loess curves using the Bisection Extremum Surface Estimator Method provided in the inflection R package[63,64]. An inflection point indicates the time at which there was a change in the shape of the loess curve (e.g., from convex to concave, or vice versa), thus informing a change in the temporal dynamics of fly navigation.

2. *Statistical modeling*

To explore whether males and females behave differently in ETLs, we contrasted the evidence supporting three competing models (statistical hypotheses) about the influence of sex on navigation behaviors, and selected the one(s) that best explained our observations. Models were fitted according to navigation decisions within each thermal landscape. For behaviors under the first navigation decision, we compared the mean proportion of fly sample (Y_1) that displayed avoidance (presence in home bottles) vs. exploration (presence in the T-System and feeding bottles) at the end of CTL or HTL experiments. For behaviors under the second navigation decision, we compared the mean proportion of explorer flies (Y_2) that exhibited thermal boldness (presence in feeding bottles) vs. shyness (presence in the T-System) at the end of CTL or HTL experiments. Then, we applied a three-steps model fitting and selection procedure following [65] to determine (1) the distribution, (2) the variance structure, and (3) the relationship between predictive variables that best described fly navigation behaviors (Appendix 2). We ranked models according to their second-order Akaike Information Criterion (*AICc*) [66,67] and selected those with *AICc* difference ($\Delta AICc$) < 2.0 relative to the top-ranked model[68]. We also calculated *AICc* weights for each candidate model, as well as the evidence ratio (ER) between the top-ranked model against alternative models, to compare the evidence supporting each model given the data [68].

3. Quantitative genetic parameters

3.1. Isofemale heritability and evolvability

To estimate sex-specific values of the isofemale heritability and evolvability of navigation behaviors in ETLs, we fitted a genetic linear mixed-effects model on the proportion of fly sample (Y_1) of females or males that displayed a given behavior. We used the VCA R package[69] to fit models via restricted maximum likelihood estimation (REML). Our general model was:

$$Y_1 = \mu + Test\ order + Obs.order + (\mu|Line) + \epsilon \quad (1)$$

where Y_1 is a vector of proportions of fly sample that exhibited a given navigation behavior (avoidance, exploration, boldness or shyness) in a given thermal landscape (CTL or HTL), $Test\ order$ is the fixed effect for the order in which the experiment was conducted within an experimental day, $Obs.order$ is the fixed effect for the position of fly samples along TDSs (and therefore order of observation during data collection), $(\mu|Line)$ is the random effect of the IL identity on the population mean value of a given Y_1 (i.e., IL-specific Y_1 value), and ϵ is the error term. From this model we estimated the total phenotypic variance ($\hat{V}_P$) for a given Y_1 and decomposed it into the among-line variance component ($\hat{V}_L$, a proxy of the total genetic variance V_G, i.e., additive, dominance, and epistatic [70]) and the residual variance component ($\hat{V}_R$).

The isofemale heritability ($\widehat{H^2}$), the proportion of the phenotypic variance ($\hat{V}_P$) that is explained by the among-line variance component ($\hat{V}_L$)[71], was estimated as $\widehat{H^2} = \hat{V}_L/(\hat{V}_L + \hat{V}_R)$. To calculate standard errors (*SE*) for $\widehat{H^2}$, we applied the delta method (based on Taylor series) that is provided in the car R package [72]. The significance of each $\widehat{H^2}$ was tested as $\widehat{H^2} - SE\big(\widehat{H^2}\big) \times Z > 0$, where Z was the quantile function of the normal distribution at $P = 0.05/16$, and 16 was the number of significance tests performed [73,74]. Additionally, we estimated the genetic-based evolvability, i.e., the ability of a population to respond to selection in the short-term given the presence of genetic variation [75,76], for each navigation behavior as the coefficient of genetic variation ($\widehat{CV_g}$) as follows:

$$\widehat{CV_g} = 100 \times \frac{\sqrt{\hat{V}_L}}{\bar{X}} \qquad (2)$$

where $\hat{V}_L$ is the among-line variance component and $\bar{X}$ is the mean value of Y_1 for a given navigation behavior [70,75].

3.2. Phenotypic and genetic correlations

Phenotypic and genetic correlations were estimated for a given navigation behavior expressed in different thermal landscapes (e.g., exploration in the CTL vs. exploration in the HTL, and so on), within sex. The phenotypic correlation ($\widehat{r_p}$) was computed as the Pearson's correlation coefficient R, using averaged Y_1 values for ILs between both thermal landscapes. To estimate the genetic correlation ($\widehat{r_g}$), we fitted the following genetic linear mixed-effects model for a given navigation behavior:

$$Y = \mu + Thermal\ landscape + (\mu|Line) + Thermal\ landscape\ \times (\mu|Line) + \epsilon \quad (3)$$

where $Thermal\ landscape$ is the fixed environmental effect (E) of the experimental thermal landscape, $(\mu|Line)$ is the random effect of the IL identity, and $Thermal\ landscape\ \times (\mu|Line)$ is the random interaction term between IL identity and the thermal landscape effect ($L \times E$). From this model we computed $\hat{V}_P$, $\hat{V}_L$, $\hat{V}_{L\times E}$, and $\hat{V}_R$, and estimated $\widehat{r_g}$ as:

$$\widehat{r_g} = \frac{\hat{V}_L}{\hat{V}_L + \hat{V}_{L\times E}} \qquad (4)$$

Finally, we estimated standard errors of $\widehat{r_g}$ using the delta method and tested its significance as $\widehat{r_g} - SE(\widehat{r_g}) \times Z > 0$, where Z was the quantile function of the normal distribution at $P = 0.05/8$ (8 was the number of significance tests performed).

Results

Temporal dynamics of fly navigation behaviors

The tendency of flies to explore or avoid the T-System (first navigation decision made by all flies in a sample) differed mainly between thermal landscapes and less between sexes (Fig. 2). When exposed to the CTL (Video S1), almost 40% of both females (Fig. 2A) and males (Fig. 2B) had left home bottles by t_{10}. Fly exploration in the CTL peaked close to t_{20} in both sexes (Fig. S2A), when on average more than 50% of flies in all samples were exploring the T-System (Fig. S2B). Between t_{20} and t_{30}, the dynamics of fly exploration and avoidance shifted in both sexes, for some flies that were exploring the T-System returned to home bottles (dashed lines in Figs. 2A, 2B). From t_{30} until the end of CTL experiments, fly exploration and avoidance reached an equilibrium and roughly half of flies in samples were exploring the T-System and the other half was in home bottles. On the other hand, flies also started exploring the HTL very quickly but apparently less than the CTL, especially males (Fig. 2C; Fig. 2D; Video S2). However, this apparent difference was caused by a quicker thermal boldness behavior displayed by explorer flies in the HTL (see below). Fly exploration and avoidance in the HTL also decelerated between t_{20} and t_{30}. But unlike the CTL, from that time fly exploration in the HTL slowly increased and peaked at the end of experiments (Fig. S2A, S2B).

The inclination of flies exploring the T-System to display either thermal boldness or shyness (second navigation decision) was also distinct between thermal landscapes, but sexes behave similarly (Fig. 3). In the CTL, almost 70% of explorer females (Fig. 3A) and males (Fig. 3B) were aggregated in the thermal gradient by t_{10} and circulated until the onset of cold CTZs. Some of these flies entered cold CTZs and fell in Cold-Induced Behavioral Impairment (CBI [42]; Video S1), but few bold flies did cold-cross successfully towards feeding bottles at that time. By t_{20} (peak of fly exploration, see above), most explorer flies were in the thermal gradient and so thermal shyness peaked at that time. But then this behavior decreased sharply until t_{30} as more explorer flies successfully cold-crossed. From t_{30} until the end of CTL experiments, both thermal boldness increased and shyness decreased at a low pace in both sexes, relative to the first half of experiments. Conversely, both explorer females (Fig. 3C) and males (Fig. 3D) were thermally bolder in the HTL than in the CTL, for hot-crosses occurred faster and at relative higher levels. Most hot-crosses occurred already by t_{20} in the HTL in both sexes. Both thermal boldness and shyness decelerated in the second half of HTL experiments, and few additional hot-crosses occurred. It is worth mentioning that some shy explorers in the HTL stayed immobile in the middle of the thermal gradient (Video S2). In very few cases, all explorer flies in the sample remained displaying such a behavior throughout the experiment without even approaching to hot CTZs nor attempting to hot-cross.

Navigation behaviors in the Cold-CTZs Thermal Landscape (CTL)

At the end of CTL experiments, females' avoidance vs. exploration were ca. 50%-50% in most samples, whereas in males' case those proportions were ca. 60%-40% respectively (Fig. 1B, left panels, solid curves). Nevertheless, sexual differences on avoidance and exploration behaviors in the CTL were negligible (Table S1). Instead, these behaviors were sensitive to the order in which flies were tested in a day, regardless of their sex (Fig. 4A). For example, on average ca. 47% of flies in all samples were in home bottles vs. 53% that were exploring any part of the system at the end of the first experiment (0100-0200 into fly photophase). But at the end of the second experiment, initiated only 30 min after ending the first, ca. 12% more flies opted for staying in home bottles, which equivalently reduced fly exploration.

When we observed the occurrence of thermal boldness and shyness in the population of isofemale lines tested, less than 25% of females and males in most fly samples were thermally bold, and a smaller fraction of flies was thermally shy (Fig. 1B, left panels, dashed curves). Smaller values for these behaviors were naturally expected at the population level, relative to avoidance and exploration, for thermal boldness and shyness are only displayed by the fraction of fly sample that explored the system. On the other hand, observing the distribution of thermal boldness and shyness from the exploratory drive of flies suggested that most explorer females and males in the CTL tended to be thermally bold (Fig. S3). Yet, differences on the occurrence of these behaviors were quite small, and neither sex of the flies or the order of experiments affected them (Table S1). On average, 53.4% of explorer flies in all samples were thermally bold and cold-crossed successfully, whereas 45.7% were shy and remained exploring the T-System (Fig. 4B). Still, thermal boldness of males was positively correlated to maximum levels of exploration, and their thermal shyness displayed the opposite pattern (Fig. S4, left panels). Finally, males and females that developed together and composed a pair of fly samples (given protocol, see above), although tested separately, displayed similar navigation behaviors in the CTL (Fig. S5, left panels).

Navigation behaviors in the Hot-CTZs Thermal Landscape (HTL)

At the end of HTL experiments, more than 50% of females in most samples were exploring the system, whereas an equivalent proportion of males in most samples avoided it (Fig. 1B, right panels, solid curves). Female exploration was on average 9.1% higher than male exploration, and so male avoidance was equivalently higher than that displayed by females. In addition, flies of both sexes were also sensitive to the order in which they were tested in the HTL (Table S1, Fig. 5A). At the end of the first experiment, on average 57% of females were exploring the system vs. 43% that were in home bottles, whereas 47% of males were exploring the system vs. 53% that were in home bottles. At the second experiment, however, fly exploration decreased by 12.5% (and so avoidance increased equivalently) in both sexes (Fig. 5A). Such an effect exacerbated the difference between male avoidance and exploration by 30.1%, relative to a 6% difference observed in the first experiment. For females, the effect of the second experiment tipped the scales in favor of avoidance, which was then ca. 12% higher than exploration.

The distribution of thermal boldness and shyness was clearly distinct at the population level, for the former occurred more uniformly among fly samples of both sexes (Fig. 1B, dashed curves). In most fly samples, ca. 25% of flies displayed thermal boldness, but cases in which this fraction exceeded 50% of the fly sample were not uncommon (see 4th quartile of boxplots in Fig. 1B, right panels). On the other hand, thermal shyness was displayed by a much smaller fraction (ca. 10%) in most fly samples. When the occurrence of thermal boldness and shyness is analyzed only for flies making those choices, it is clear that most explorer flies in the HTL were thermally bold regardless of their sex (Fig. S3). Also, both females and males exploring the HTL were sensitive to the order in which experiments were performed in a day (Fig. 5B; Table S1). At the end of the first experiment, ca. 77% of explorers hot-crossed towards feeding bottles, whereas the proportion of explorers that remained in the thermal gradient was ca. 55.1% lower. In the second experiment, shyness increased 14.1% (and so thermal boldness decreased equivalently), and such an effect reduced the difference between thermal boldness and shyness to 26.9%. Furthermore, only thermal boldness of females was positively associated to their maximum level of exploration (Fig. S4, right panels). Unlike the CTL, females and males that shared the same developmental environment displayed similar patterns of avoidance and exploration of the HTL, but not thermal boldness or shyness (Fig. S5, right panels).

Quantitative genetics of fly navigation behaviors

Broadly, navigation behaviors varied among ILs within each thermal landscape, and within-line sex variation seemed negligible (Fig. S6). However, our variance component analyses indicated that the among-line variance of almost all navigation behaviors in the CTL was very low and close to zero (Table 1), whereas the residual variance accounted for 51-88% of the total phenotypic variance (Appendix 2). Consequently, most navigation behaviors in the CTL displayed low, non-significant values of isofemale heritability, whereas evolvability was less than 36%. Only thermal boldness of males displayed a significant and moderate isofemale heritability value, and a moderate evolvability (Table 1).

In contrast, with the exception of thermal shyness of females, most navigation behaviors in the HTL did display substantial among-line variation (Table 1). Specifically, the among-line variance component accounted for 48-68% of the total phenotypic variance. Consequently, most navigation behaviors in the HTL displayed significant levels of isofemale heritability in the range of 0.485 – 0.680. Evolvability estimates ranged from 28.34 – 77.91%, with no obvious association with heritability estimates. For instance, thermal shyness of females and thermal boldness of males displayed clear differences in isofemale heritability (0.246 vs. 0.680, respectively, and non-significant in females), yet comparable evolvability (55.05% vs. 57.23%, respectively).

Regarding sex-specific associations in navigation behaviors between thermal landscapes, no phenotypic correlation was observed (Table 2, Fig. S7). Furthermore, both the among-line and the line × thermal landscape variance components were very low and close to zero in all cases. On the other hand, the residual variance component alone accounted for more 52-78% of the total phenotypic variance (Appendix 2). Then, no navigation behavior was genetically correlated between thermal landscapes. This is an interesting outcome, considering most behaviors (but not thermal boldness) displayed genetic correlation estimates higher than 0.6. But in all cases the size of the standard error was at least 45.5% the size of the respective estimate.

Discussion

This investigation furthers our understanding about the decision rules followed by small motile ectothermic animals when navigating through extreme thermal landscapes (ETLs). By using two configurations of the so-called module of Thermal Decision Systems (TDSs) and an isofemale line design, we quantified the phenotypic and genotypic variation of navigation behaviors of fruit flies in ETLs within our studied population. We confirmed that, when allowed to navigate through the T-

System (region of TDSs were ETLs were set), flies within samples split into those who explored it and those who avoided it. The fraction of avoiders vs. explorers formed a continuum along which fly samples were distributed, with mean population-level responses fluctuating around 50%-50% across experimental groups. Avoider flies in particular, chose to stay in home bottles, the known and spatiotemporally constant thermal environment, so never faced a thermal risk. Then, avoidance of novelty, understood here as the lack of an exploratory drive, constitutes the most conservative strategy displayed by flies in ETLs. On the other hand, we observed that most flies that explored the T-Systems engaged in thermal risks and crossed through Critical Temperature Zones (CTZs), so thermal boldness occurred more frequently than shyness at the population level. Such a tendency occurred in both ETLs, but it was higher in the Hot-CTZs Thermal Landscape (HTL). This evidence supports, under our first hypothesis, a *behavior-is-opportunity* strategy in our studied population regarding thermal risk-taking behaviors in ETLs, especially towards extreme heat.

A natural question that follows is: opportunity to what? Our data do not answer this question, but ecological and evolutionary theory open room for some (and just that so far) potential speculations that might guide formal tests. Ecologically, thermal boldness might open opportunities for explorer individuals to exploit resources (e.g., food, mates) available at or across extreme thermal conditions [42,77]. Persistence in thermal exploration and boldness in ETLs (e.g., see Video S2 in [42]) may also drive short-term physiological adjustments (e.g., thermal hardening) [15]. Moreover, if thermally bold individuals reproduce in novel thermal environments, transgenerational thermal adjustments in offspring may occur and enhance offspring fitness [78,79]. Evolutionarily, these factors may expand animal distributions into new adaptive zones [80,81] and facilitate scenarios of thermal niche divergence, including behaviorally-driven evolution of thermal physiology. This possibility is particularly intriguing, considering thermoregulatory behaviors are mostly seen as “brakes” of physiological evolution in ectothermic animals [82,83]. Comparative studies in, for example, populations at the core/native vs. edge/invasive ranges, may further our comprehension on the ecological and evolutionary implications of navigation behaviors of small ectothermic animals in ETLs.

We partially confirmed our second hypothesis, for navigation behaviors of both females and males displayed high genetic variation, heritability, and evolvability only in the HTL. On the contrary, navigation behaviors of fruit flies in the CTL were characterized by low genetic variation, non-significant heritability, and low evolvability. These outcomes are surprising and contrasts with the vast amount of evidence pointing out to a strongly constrained evolutionary potential of heat tolerance vs. a more labile cold tolerance in fruit flies and other lineages of ectothermic animals [38–40,50]. Yet, that navigation behaviors in hot thermal environments have high genetic-based evolutionary potential in the short term [76] does not mean adaptive evolution will necessarily occur, but that there is genetic variation for selection to operate [75,84]. However, it is uncertain at this moment what selection contexts would favor adaptive evolution of navigation behaviors in hot thermal environments and in what direction it would occur. Similarly, the low genetic-based evolutionary potential of navigation behaviors in the cold does not preclude them to evolve [85]. Alternative sources of phenotypic variation, like epigenetic [86], as well as social and stochastic developmental effects [47,87,88], may also be substrate for the evolution of non-genetically heritable traits [85,89]. In this context, fly development may be a relevant aspect influencing navigation behaviors in the cold, as females and males that developed together behave similarly in the CTL.

Although our main aim was to study navigation behaviors in extreme cold or heat in isolation, it was clear that fruit flies of both sexes explored quicker and were bolder in the HTL than in the CTL. These patterns contrast with previous observations when flies were exposed to both extreme cold and heat in the same thermal landscape [42]. Then, the configuration of the thermal landscape modulates navigation behaviors of fruit flies in complex ways, beyond what we might expect from the thermal risk asymmetry between extreme cold and heat ([35]; see Fig. 8 in [42]). In fact, navigation behaviors in extreme cold vs. heat are perhaps completely different traits controlled by different and independent loci, as indicated by the lack of both phenotypic and genetic correlations in any navigation behavior between ETLs [90,91]. This possibility is further supported by the nature of the thermosensory system of fruit flies. Coding of cold and heat stimuli (either noxious or innocuous) in the fly brain is mediated by different thermoreceptors at the molecular, cellular, and anatomical levels [51,52,92]. Silencing cellular (e.g., hot- or cold-sensitive neurons) or molecular (e.g., Transient Receptor Potential channels, Ionotropic Receptors) thermoreceptors may shed light into the thermosensory control of navigation behaviors of fruit flies in ETLs [44,52].

Exploration in both ETLs, and thermal boldness in the HTL, decreased during the second experiments we performed in a day, initiated only 30 min after the first experiments ended. This effect was evident in flies of both sexes, and there was no difference in starvation time between experiments. Odor clues (resource- and pheromone-linked) may have differed between the first and second experiments. If ETLs affected food odor plumes (e.g., reduced in the CTL, enhanced in the HTL [93]), these had to be more pronounced during the second experiments simply because the thermal bath was on longer. In addition, explorer flies tested in the first experiments may have left chemical signals in T-Systems that were available for flies tested in the second experiments. But if exploration and thermal boldness were just a consequence of odor-tracking behaviors, they would have increased (not decreased) during the second experiments [94]. Instead, it is likely that navigation behaviors in ETLs have a circadian component. Although both experiments were conducted during the morning peak of activity, it is possible flies were more active during the first experiments shortly after lights-on [95]. Yet, activity alone would not explain higher exploration and boldness in ETLs, as no correlation seem to exist between these variables (Wiil et al., Unpublished data). Then, cognitive processes underlying fly exploration and thermal boldness might also be under circadian control to allow flies modulate navigation behaviors in response to natural daily temperature cycles [96].

Most behavioral studies in fruit flies use only one sex in experiments [97], which hinders our comprehension of sex-related behavioral differences. In this study we observed that navigation behaviors of fruit flies in ETLs and their genetic-based evolutionary potential varied little between the sexes. Such lack of sex effect is by no mean irrelevant, and suggest that both virgin males and females share and modulate similarly the genetic, thermosensory, and neural components of their navigation behaviors in ETLs. We did observe that virgin females tended to explore more ETLs than virgin males, especially the HTL. While higher exploratory activity has been observed before for females of *D. melanogaster* [98], males display territorial and aggressive behaviors [99,100]. Then, sex differences in exploratory activity in fruit flies have been associated to search for oviposition sites by females vs. defense of territories by males [98]. It is yet to determine, though, if (and how) mating would affect navigation behaviors of female and male fruit flies.

Finally, it is clear that navigation behaviors in both ETLs were highly variable (at least phenotypically) in our studied population of fruit flies. Given this, it is tempting to ask: what historical factors may have contributed to shaping the extant behavioral variation within our studied population? Honestly, we (currently) do not know, and the data presented here do not answer that question either. Yet, life history theory predicts that polybehavioral populations result from a trade-off between current and future reproduction [101–103]. In this context, disruptive selection acting on individuals with different fitness expectations would give rise to the evolution of continuous variation in animal personalities within a population [103]. Accordingly, navigation behaviors exhibited by fruit flies in ETLs may correlate with a continuum of fitness expectations, with avoider flies at one end prioritizing future fitness (e.g., long-term survival and reproduction), and thermally bold flies at the other end emphasizing immediate survival (e.g., by getting food). However, thermal boldness may also exhibit stereotypical characteristics in few individuals (e.g., Video S3), without any apparent link with an immediate benefit.

Conclusions

Although patterns of phenotypic and genotypic variation reported here relate only to our studied population, it is clear that the configuration of the thermal landscape modulates navigation behaviors of fruit flies, as well as their genetic-based evolutionary potential. Extreme cold and heat are two different ecological contexts, posing different challenges to fly physiology (bottom-up) and activating different thermosensory pathways in the fly brain (top-down). Behaviorally, fruit flies navigate differently through extremely cold or hot thermal landscapes, displaying complex strategies beyond simply thermophobic responses. Navigation behaviors in extreme cold or hot thermal landscapes are not genetically constrained, but it is unclear whether other constraints (e.g., developmental) would prevent their independent evolution. So far, our knowledge on the navigation decisions and behaviors employed by small motile ectothermic animals in ETLs is virtually concentrated on the "how's?". Yet, a comprehensive understanding of these aspects of thermal biology requires also addressing the "why's", including those historical [104]. In this context, a closer dialogue between ecological physiologists and behavioral ecologists may render very fruitful. For instance, navigation behaviors in ETLs may be inspected *per* se in terms of animal personalities [45], as well as potential physiological correlates (e.g., metabolic rate, basal thermal tolerance, acclimation capacity). In this context, the pace-of-life syndrome offers an interesting framework to integrate these aspects [46,105]. On the other hand, combining selection experiments and genome-wide association may prove useful to test the overall evolutionary potential of navigation behaviors of fruit flies in ETLs. Furthermore, the fact that distant lineages also explore voluntarily ETLs and display thermal boldness [43] paves the way for comparative studies on the evolution of such behaviors along the insect phylogeny, and opens room for their scrutiny in ectothermic vertebrates with higher cognitive capabilities.

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Data and code availability

The data and code supporting the results of this study will be fully available in a public repository after publication.

Author contributions

GAAC, JGS, CAN and VL conceived the study. GAAC standardized and conducted the experiments. GAAC performed the data analysis. PDR assisted with data analysis and interpretation. JGS, CAN and VL supervised the research. GAAC, JGS, and VL acquired funding. GAAC wrote the first draft of the manuscript. All authors contributed to revisions and approved the final version of the manuscript.

Ethics declarations

Competing interests

The authors declare no competing interests.

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TABLES

Table 1. Estimates for mean and standard errors, among-line variance ($\hat{V}_L$, as a proxy of the total genetic variance V_G), residual variance ($\hat{V}_R$), isofemale heritability ($\widehat{H^2} \pm SE$), and genetic-based evolvability ($\widehat{CV}_g$, %) of navigation behaviors of a population of fruit flies (*D. melanogaster*) in extreme thermal landscapes, per sex. 95% confidence intervals (LCL: lower confidence limit; UCL: upper confidence limit; *constrained to be ≥ 0) for variance components are provided. Values of isofemale heritability in bold are significantly different from 0 at P=0.05⁄16 (see text). Since the proportion of avoidance = 1 - the proportion of exploration, values of variances and isofemale heritability are the same for both behaviors. However, the same is not true for evolvability values, for avoidance and exploration differed in their mean phenotypic value (see text for calculation of $\widehat{CV}_g$).

Thermal landscape	**Behavior**	**Sex**	**Mean ± SE**	$\hat{V}_L$			$\hat{V}_R$			$\widehat{H^2}$ **± SE**	$\widehat{CV}_g$**(%)**
				Estimate	**LCL**	**UCL**	**Estimate**	**LCL**	**UCL**		
Cold-CTZs (CTL)	Avoidance / Exploration	Females	0.52 ± 0.02 / 0.48 ± 0.02	0.013	0*	0.026	0.022	0.015	0.038	0.362 ± 0.147	21.71 / 23.84
		Males	0.54 ± 0.02 / 0.46 ± 0.02	0.013	0*	0.026	0.020	0.013	0.035	0.384 ± 0.152	20.94 / 24.35
	Boldness	Females	0.26 ± 0.02	0.009	0*	0.019	0.018	0.012	0.030	0.333 ± 0.149	35.79
		Males	0.26 ± 0.02	0.015	0.002	0.028	0.016	0.010	0.027	**0.485 ± 0.140**	46.45
	Shyness	Females	0.21 ± 0.02	0.002	0*	0.009	0.018	0.012	0.030	0.118 ± 0.149	23.14
		Males	0.20 ± 0.01	0.003	0*	0.007	0.011	0.007	0.018	0.207 ± 0.161	25.86
		Females	0.50 ± 0.03 /	0.028	0.005	0.051	0.030	0.020	0.049	**0.486 ± 0.130**	33.62 / 33.27

Hot-CTZs (HTL)	Avoidance / Exploration		0.50 ± 0.03							
		Males	0.58 ± 0.03 / 0.42 ± 0.03	0.027	0.005	0.050	0.024	0.015	0.042	**0.536 ± 0.135**	28.34 / 39.73
	Boldness	Females	0.38 ± 0.03	0.033	0.007	0.058	0.031	0.021	0.051	**0.510 ± 0.126**	47.22
		Males	0.30 ± 0.03	0.030	0.008	0.051	0.014	0.009	0.025	**0.680 ± 0.104**	57.23
	Shyness	Females	0.12 ± 0.02	0.004	0*	0.010	0.014	0.009	0.022	0.246 ± 0.148	55.05
		Males	0.12 ± 0.02	0.008	0.001	0.015	0.009	0.006	0.015	**0.485 ± 0.144**	77.91

Table 2. Phenotypic ($\widehat{r_p}$, estimated as the Pearson's correlation coefficient *R*, Fig. S7) and genetic correlations ($\widehat{r_g}$) in navigation behaviors of fruit flies between thermal landscapes, per sex. Estimates for the among-line variance ($\widehat{V}_L$), the variance of the interaction between the isofemale lines and the experimental thermal landscape ($\widehat{V}_{L\times E}$), as well as their 95% confidence intervals are provided. All estimated parameters for avoidance and exploration are the same for within sexes for the same reason explained in Table 1. No *R* or $\widehat{r_g}$ value was significantly different from 0 at P=0.05⁄8 (see text).

Pair of traits / Thermal landscape	**Sex**	**R**	**$\widehat{V}_L$**			**$\widehat{V}_{L\times E}$**			**$\widehat{V}_R$**			**$\widehat{r_g} \pm SE$**
			Estimate	**LCL**	**UCL**	**Estimate**	**LCL**	**UCL**	**Estimate**	**LCL**	**UCL**	
Avoidance (Exploration) - CTL vs. Avoidance (Exploration) - HTL	Females	0.37	0.012	0*	0.025	0.005	0*	0.017	0.039	0.030	0.054	0.689 ± 0.321
	Males	0.41	0.012	0*	0.025	0.008	0*	0.019	0.028	0.021	0.040	0.600 ± 0.273
Boldness - CTL vs. Boldness - HTL	Females	0.22	0.006	0*	0.018	0.011	0*	0.026	0.033	0.025	0.046	0.335 ± 0.343
	Males	0.23	0.007	0*	0.020	0.014	0*	0.028	0.023	0.017	0.033	0.344 ± 0.284
Shyness - CTL vs. Shyness - HTL	Females	0.34	0.003	0*	0.008	0.001	0*	0.006	0.016	0.012	0.022	0.716 ± 0.430
	Males	0.37	0.003	0*	0.007	0.002	0*	0.006	0.013	0.010	0.018	0.638 ± 0.390

FIGURE LEGENDS

Figure 1. Navigation behaviors of fruit flies (*Drosophila melanogaster*) in extreme thermal landscapes (ETLs). A) One replicate of the Thermal Decision System depicting the navigation decisions fruit flies face and the resulting behaviors (see main text). "IN" and "OUT" indicate water flow through the system from and to the thermal bath. B) Distribution (raincloud plots) of navigation behaviors in our studied population in each thermal landscape, by sex. The x-axis displays the proportion of flies in a sample that at the end of experiments exhibited a given behavior (Y_1 in the main text), and each data point is one fly sample.

Figure 2. Temporal dynamics of exploration (blue) and avoidance (red) behaviors of fruit flies in ETLs. A) Females' exploration and avoidance in the Cold-CTZs Thermal Landscape (CTL). B) Males' exploration and avoidance in the CTL. C) Females' exploration and avoidance in the Hot-CTZ Thermal Landscape (HTL). D) Males' exploration and avoidance in the HTL. Time $0'$ (t_0) marks the onset of experiments when all flies in samples were in home bottles (avoidance = 1, exploration = 0). Data collection started at t_{10} and continued at a 10-min interval. The y-axis presents Y_1 values for avoidance or exporation. Curves (shadowed areas for confidence intervals) are loess fits to the data (points for fly samples). The black dashed line is the inflection point of both exploration (concave to convex) and avoidance (convex to concave) curves, which signals a change in the temporal dynamics of these behaviors (see main text).

Figure 3. Temporal dynamics of thermal boldness (green) and shyness (purple) of fruit flies exploring ETLs. A) Females' thermal boldness and shyness in the CTL. B) Males' thermal boldness and shyness in the CTL. C) Females' thermal boldness and shyness in the HTL. D) Males' thermal boldness and shyness in the HTL. The y-axis presents the proportion of flies exploring the T-System that either crossed a cold (CTL) or hot (HTL) Critical Temperature Zone (thermal boldness), or stayed in the T-

System (shyness) at a given time (i.e., Y_2 in the main text). Most figure details as in Fig. 2. Dashed purple lines denotes inflection points (concave to convex) for the loess curve on thermal shyness data only.

Figure 4. Navigation behaviors of fruit flies in the Cold-CTZs Thermal Landscape (CTL). A) Behaviors resulting from the first navigation decision, according to the time into fly photophase in which experiments were conducted. B) Behaviors resulting from the second navigation decision. Mean (black diamonds) and raw values (grey circles, each data point is one fly sample) are displayed. Boxes represent the 1st, 2nd (i.e., median, horizontal line), and 3rd quartiles, while whiskers extend from hinges ± 1.5× IQR (the inter-quartile range).

Figure 5. Navigation behaviors of fruit flies in the Hot-CTZs Thermal Landscape (HTL), according to the time into fly photophase in which experiments were conducted. A) Behaviors resulting from the first navigation decision. B) Behaviors resulting from the second navigation decision. Figure details as in Fig. 4.

FIGURES

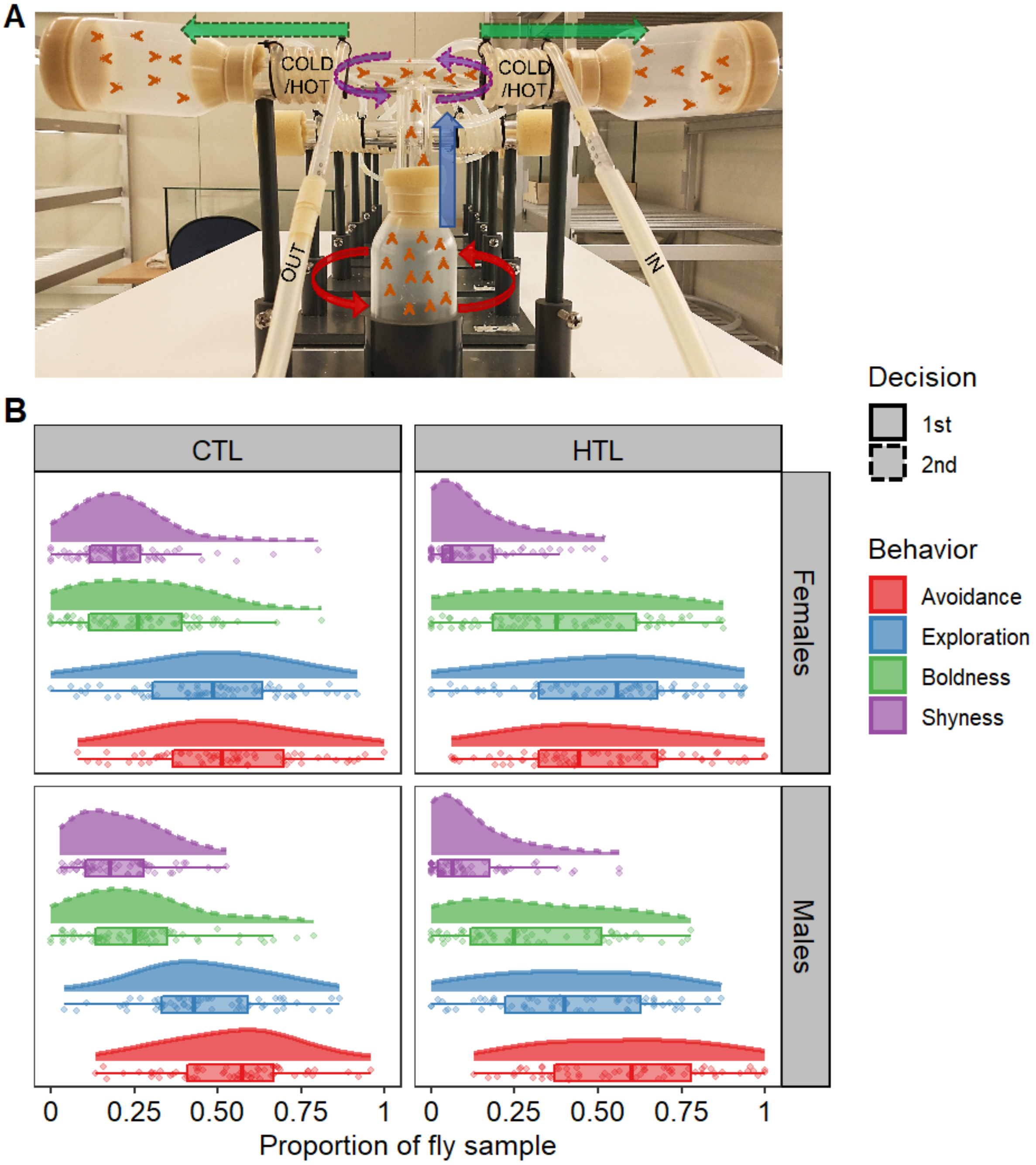


Figure 1

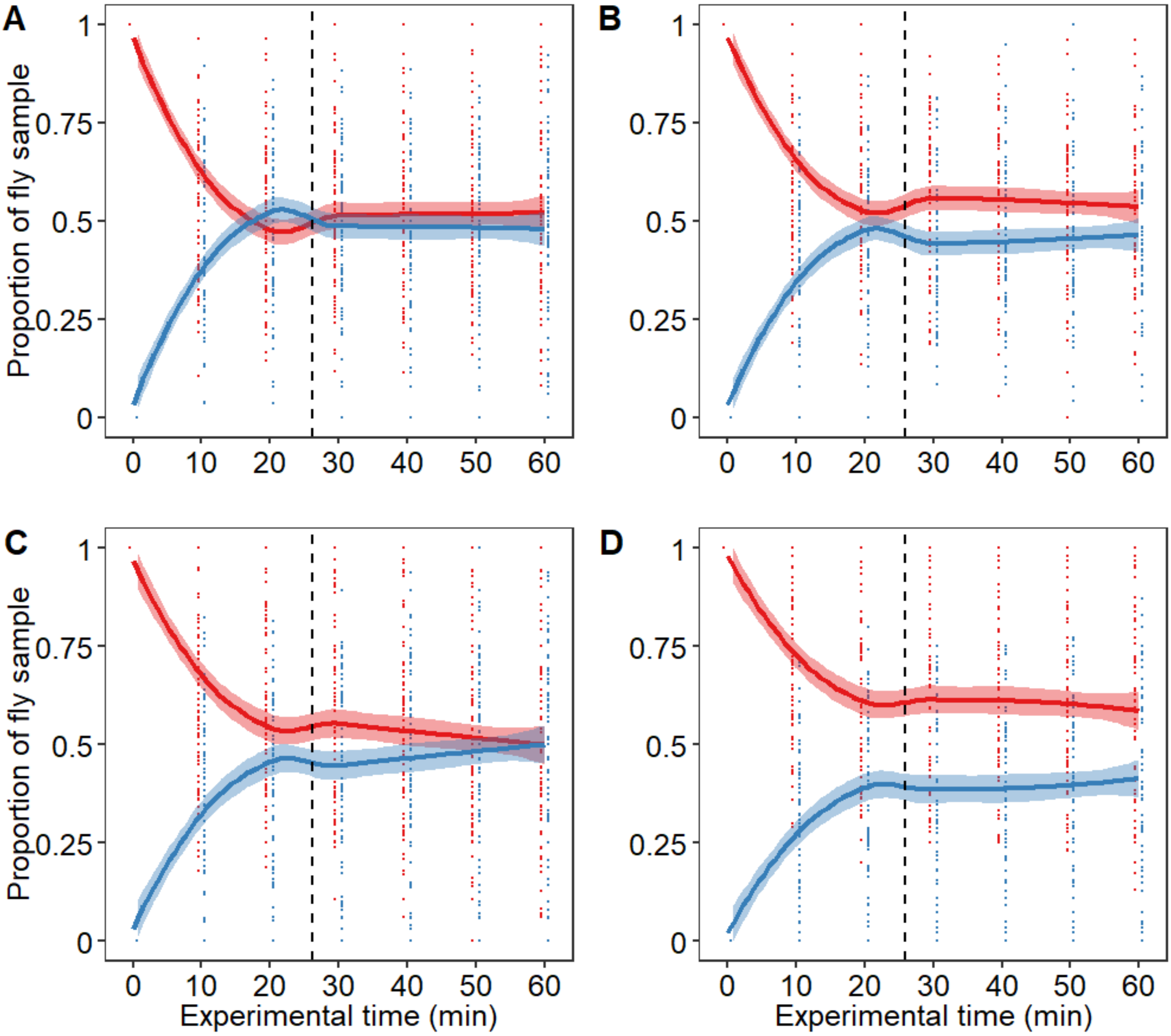

A
B
C
D
Proportion of fly sample
Proportion of fly sample
1
0.75
0.5
0.25
0
0
10
20
30
40
50
60
Experimental time (min)
Experimental time (min)

Figure 2.

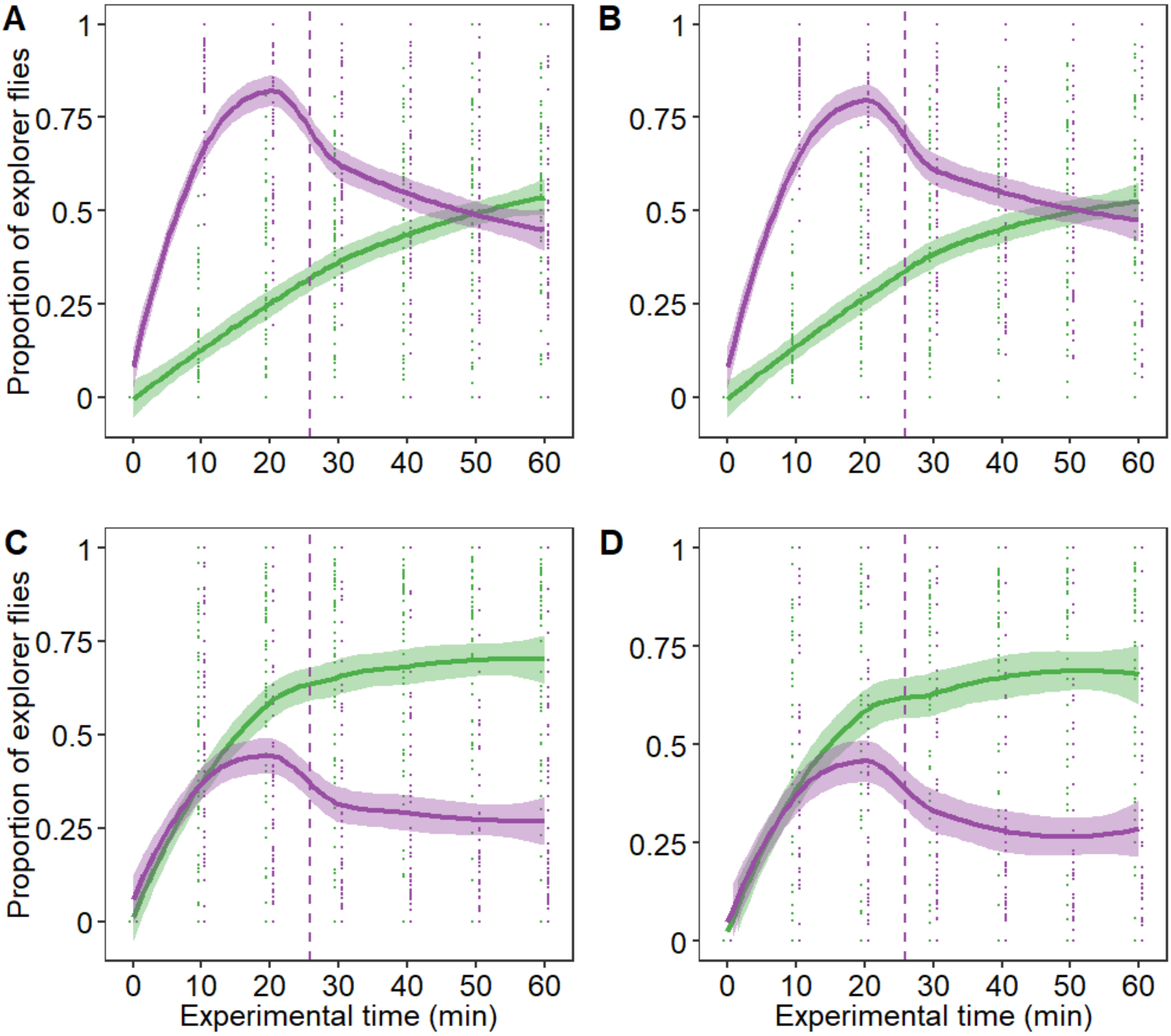

A
B
C
D
Proportion of explorer flies
Experimental time (min)
0
0.25
0.5
0.75
1
0
10
20
30
40
50
60

Figure 3.

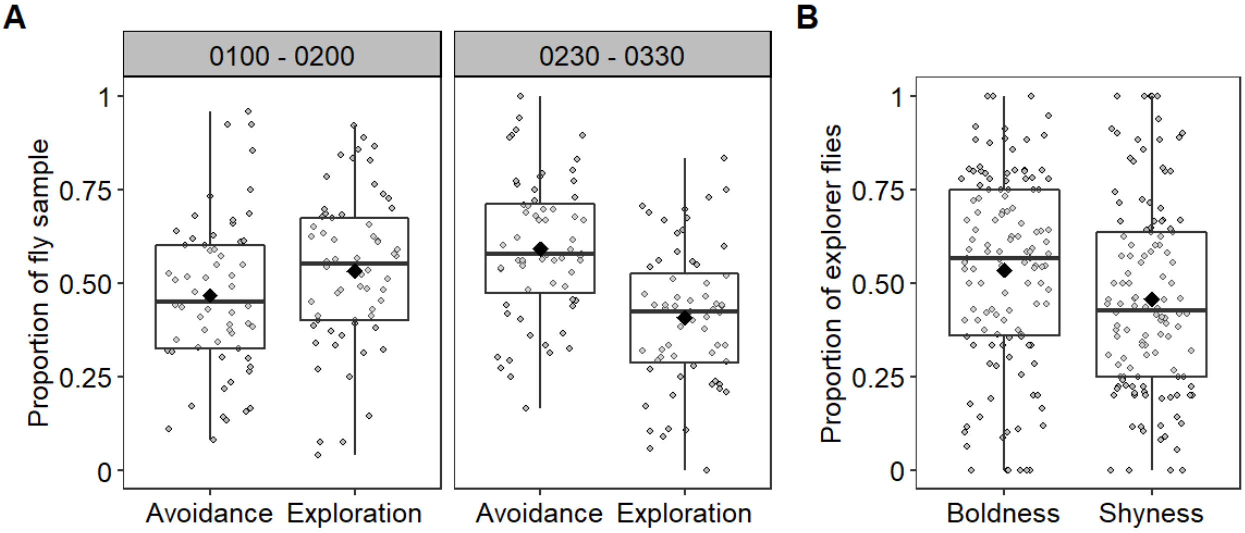

A
0100 - 0200
0230 - 0330
Proportion of fly sample
1
0.75
0.50
0.25
0
Avoidance
Exploration
Avoidance
Exploration
B
Proportion of explorer flies
1
0.75
0.50
0.25
0
Boldness
Shyness

Figure 4.

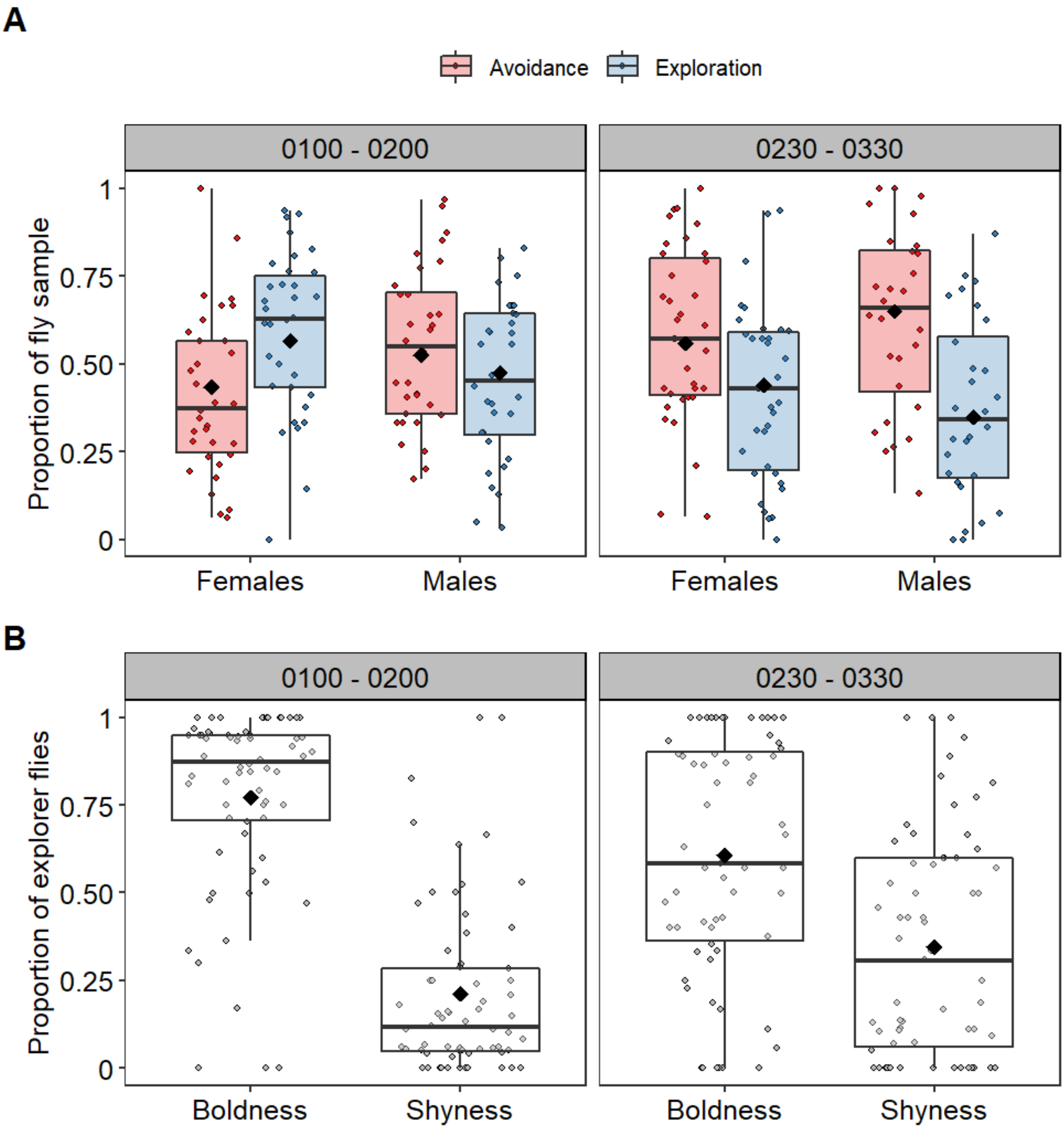

A
Avoidance
Exploration
0100 - 0200
0230 - 0330
Proportion of fly sample
1
0.75
0.50
0.25
0
Females
Males
Females
Males
B
0100 - 0200
0230 - 0330
Proportion of explorer flies
1
0.75
0.50
0.25
0
Boldness
Shyness
Boldness
Shyness

Figure 5.

Nature Ecology & Evolution

SUPPLEMENTARY INFORMATION

Navigation behaviors of fruit flies in spatially explicit thermal landscapes

Gustavo A. Agudelo-Cantero[1,2,*], Jesper Givskov Sørensen[2], Palle Duun Rohde[3], Carlos A. Navas[1†], Volker Loeschcke[2†].

[1] Department of Physiology, Institute of Biosciences, University of São Paulo, Rua do Matão 101, Tv 14, 05508-090 São Paulo, Brazil.

[2] Department of Biology - Genetics, Ecology and Evolution, Faculty of Natural Sciences, Aarhus University. Ny Munkegade 116, 8000 Aarhus, Denmark.

[3] Department of Chemistry and Bioscience, Faculty of Engineering and Science, Aalborg University. Frederik Bajers Vej 7H, 9220 Aalborg, Denmark.

[*] Corresponding author: gustavo.agudelo@ib.usp.br, gustavo.a.agudelo@gmail.com

[†]CAN and VL are both senior authors.

SUPPLEMENTARY TABLES

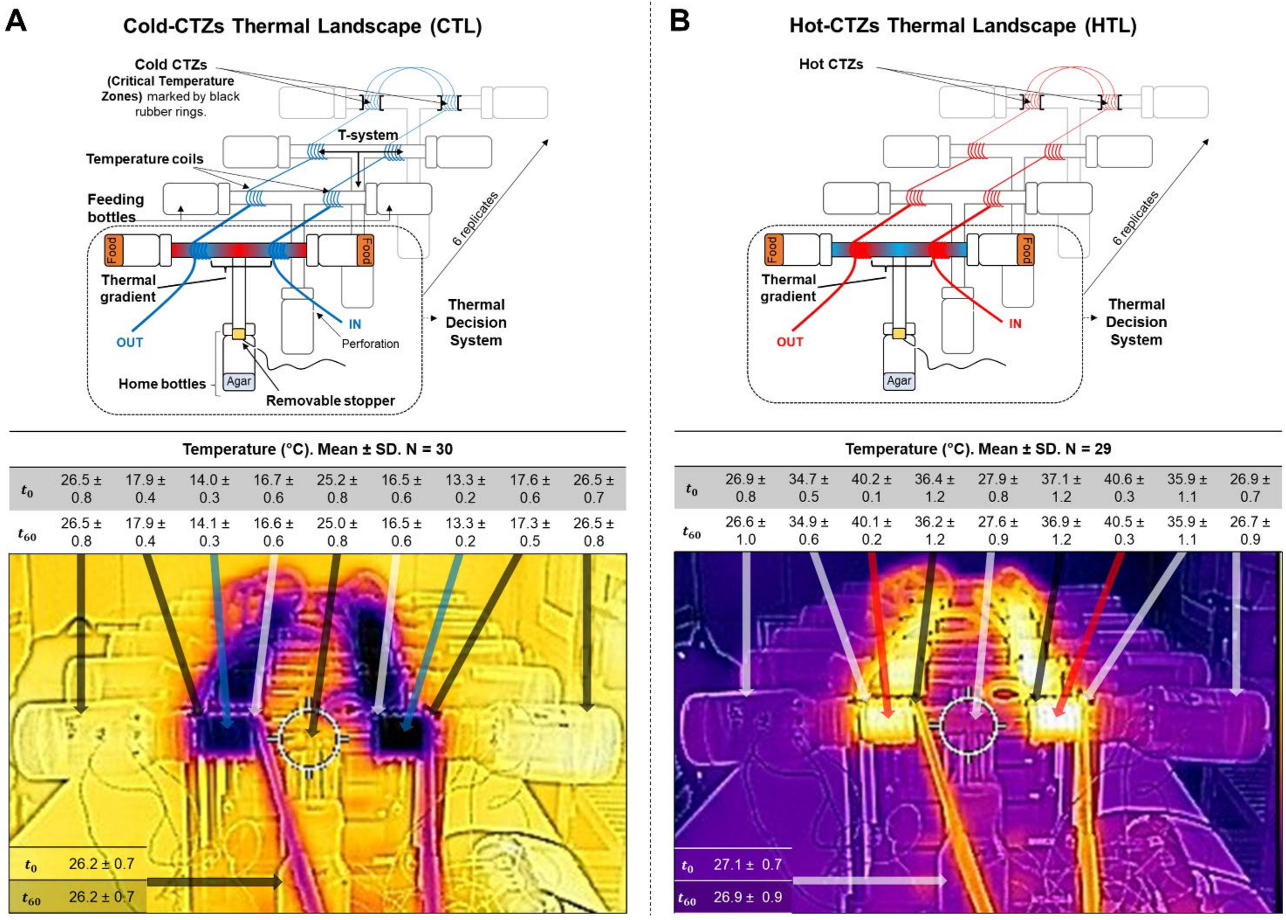
Table S1. Statistical models (ultimate comparison, see Appendix 2) to explain variation in navigation behaviors of fruit flies (*Drosophila melanogaster*) in extreme thermal landscapes (ETLs). Within each thermal landscape (CTL: Cold-CTZs Thermal Landscape; HTL: Hot-CTZs Thermal Landscape), we compared the proportion of the fly sample (Y_1) that displayed avoidance or exploration, or the proportion of explorer flies (Y_2) that displayed thermal boldness or shyness. Selected models in bold. *k* is the number of parameters estimated by the model. *AICc* is the value for the second-order Akaike information criterion. *ΔAICc* is the difference in *AICc* values between the top-ranked model and an alternative candidate model. *AICc weight* is the evidence in favor of a candidate model, among the set of models under comparison, to explain the data. *ER* is the evidence ratio of the *AICc* weight between the top-ranked model and an alternative candidate model.

Thermal landscape	**Comparison / Model**	***k***	***AICc***	***ΔAICc***	***AICc weight***	***ER***
Cold-CTZs Thermal Landscape (CTL)	Avoidance vs. exploration					
	$\boldsymbol{Y_1 = \mathit{Behavior} + (\mathit{Behavior} \mid \mathit{Test\ order}) + \varepsilon}$	6	-93.8	0.0	0.6	
	$Y_1 = \mathit{Behavior} + \mathit{Sex} + (\mathit{Behavior} \mid \mathit{Test\ order}) + \varepsilon$	7	-91.7	2.1	0.2	2.9
	$Y_1 = \mathit{Behavior} \times \mathit{Sex} + (\mathit{Behavior} \mid \mathit{Test\ order}) + \varepsilon$	8	-91.4	2.4	0.2	3.3
	Thermal boldness vs. shyness					
	$\boldsymbol{Y_2 = \mathit{Behavior} + \varepsilon}$	3	32.5	0.0	0.7	
	$Y_2 = \mathit{Behavior} + \mathit{Sex} + \varepsilon$	4	34.5	2.0	0.2	2.7
	$Y_2 = \mathit{Behavior} \times \mathit{Sex} + \varepsilon$	5	36.3	3.8	0.1	6.8

Hot-CTZs Thermal Landscape (HTL)	Avoidance vs. exploration					
	$\boldsymbol{Y_1 = \mathit{Behavior} \times \mathit{Sex} + (\mathit{Behavior} \mid \mathit{Test\ order}) + \varepsilon}$	8	9.6	0.0	0.9	
	$Y_1 = \mathit{Behavior} + (\mathit{Behavior} \mid \mathit{Test\ order}) + \varepsilon$	6	14.7	5.1	0.1	12.6
	$Y_1 = \mathit{Behavior} + \mathit{Sex} + (\mathit{Behavior} \mid \mathit{Test\ order}) + \varepsilon$	7	16.8	7.2	0.0	36.2
	Thermal boldness vs. shyness					
	$\boldsymbol{Y_2 = \mathit{Behavior} + (\mathit{Behavior} \mid \mathit{Test\ order}) + \varepsilon}$	6	113.5	0.0	0.7	
	$Y_2 = \mathit{Behavior} + (\mathit{Behavior} \mid \mathit{Test\ order}) + \mathit{Sex} + \varepsilon$	7	115.6	2.1	0.2	2.9
	$Y_2 = \mathit{Behavior} \times \mathit{Sex} + (\mathit{Behavior} \mid \mathit{Test\ order}) + \varepsilon$	8	117.2	3.7	0.1	6.4

SUPPLEMENTARY FIGURES

Figure S1. Experimental thermal landscapes, modified from Navas et al. (2022). Only one thermal bath was coupled to the module of Thermal Decision Systems (TDSs) to circulate either cold (A) or hot (B) water. (Upper panels) Schematic for each thermal landscape configuration, displaying 4 out of 6 replicates of the TDS module and its main parts (detailed only in A). (Lower panels) Thermographic images of each thermal landscape, with temperature data in key spots of the first TDS at the onset (t_0) and end (t_{60}) of behavioral experiments. Blue and red arrows in the middle of Critical Temperature Zones (CTZs, cold or hot) denote the area in which temperature of the system is most extreme. CTZs then may act as thermal barriers and present thermal risks for fly performance and survival. *N* is the number of experiments performed in each thermal landscape. Room temperature was 26 ± 1°C in all experiments.



Temperature (°C). Mean ± SD. N = 30

t_0	26.5 ± 0.8	17.9 ± 0.4	14.0 ± 0.3	16.7 ± 0.6	25.2 ± 0.8	16.5 ± 0.6	13.3 ± 0.2	17.6 ± 0.6	26.5 ± 0.7
t_{60}	26.5 ± 0.8	17.9 ± 0.4	14.1 ± 0.3	16.6 ± 0.6	25.0 ± 0.8	16.5 ± 0.6	13.3 ± 0.2	17.3 ± 0.5	26.5 ± 0.8

t_0	26.2 ± 0.7
t_{60}	26.2 ± 0.7

Temperature (°C). Mean ± SD. N = 29

t_0	26.9 ± 0.8	34.7 ± 0.5	40.2 ± 0.1	36.4 ± 1.2	27.9 ± 0.8	37.1 ± 1.2	40.6 ± 0.3	35.9 ± 1.1	26.9 ± 0.7
t_{60}	26.6 ± 1.0	34.9 ± 0.6	40.1 ± 0.2	36.2 ± 1.2	27.6 ± 0.9	36.9 ± 1.2	40.5 ± 0.3	35.9 ± 1.1	26.7 ± 0.9

t_0	27.1 ± 0.7
t_{60}	26.9 ± 0.9

Figure S2. Maximum exploration of male or female fruit flies in extreme thermal landscapes. A) Density plots for the time at which the proportion of flies within a sample that were exploring the T-System was maximum. Dashed lines at peaks of density curves denote the time for maximum exploration in most fly samples. B) Maximum proportion of flies in a sample that explored the T-System. Mean (black diamonds) and raw values (grey circles, each data point is one fly sample) are displayed. Boxes represent the 1st, 2nd (i.e., median, horizontal line), and 3rd quartiles, while whiskers extend from hinges ± 1.5 × IQR (the inter-quartile range).

Figure S2

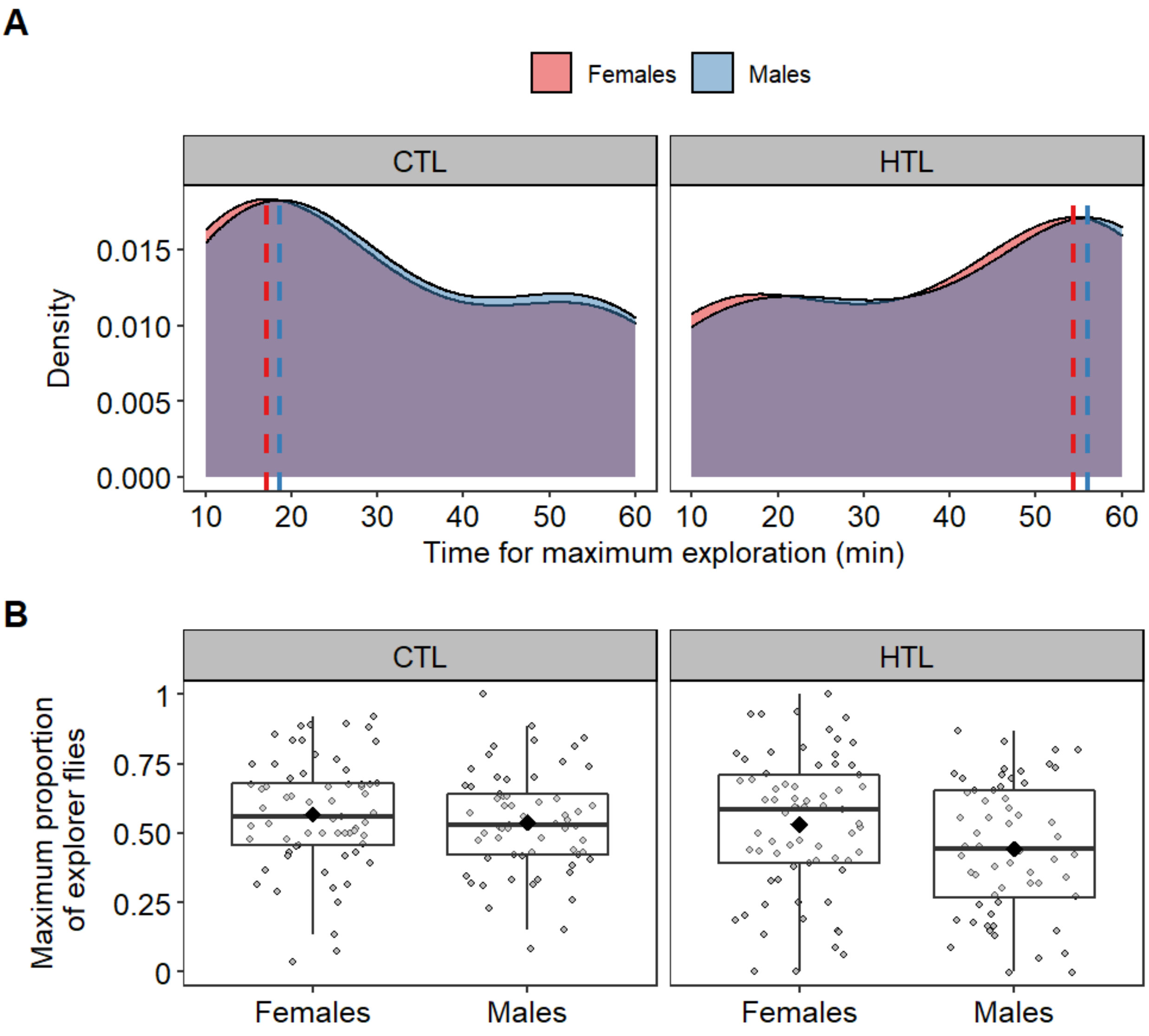


Figure S3. Distribution (raincloud plots) of thermal boldness and shyness displayed by male or female fruit flies (*Drosophila melanogaster*) exploring voluntarily extreme thermal landscapes (ETLs). The *x*-axis is the proportion of explorer flies in a sample that at the end of experiments had either crossed a CTZ (thermal boldness) or stayed in the T-system (thermal shyness). Each data point is one fly sample.

Figure S3

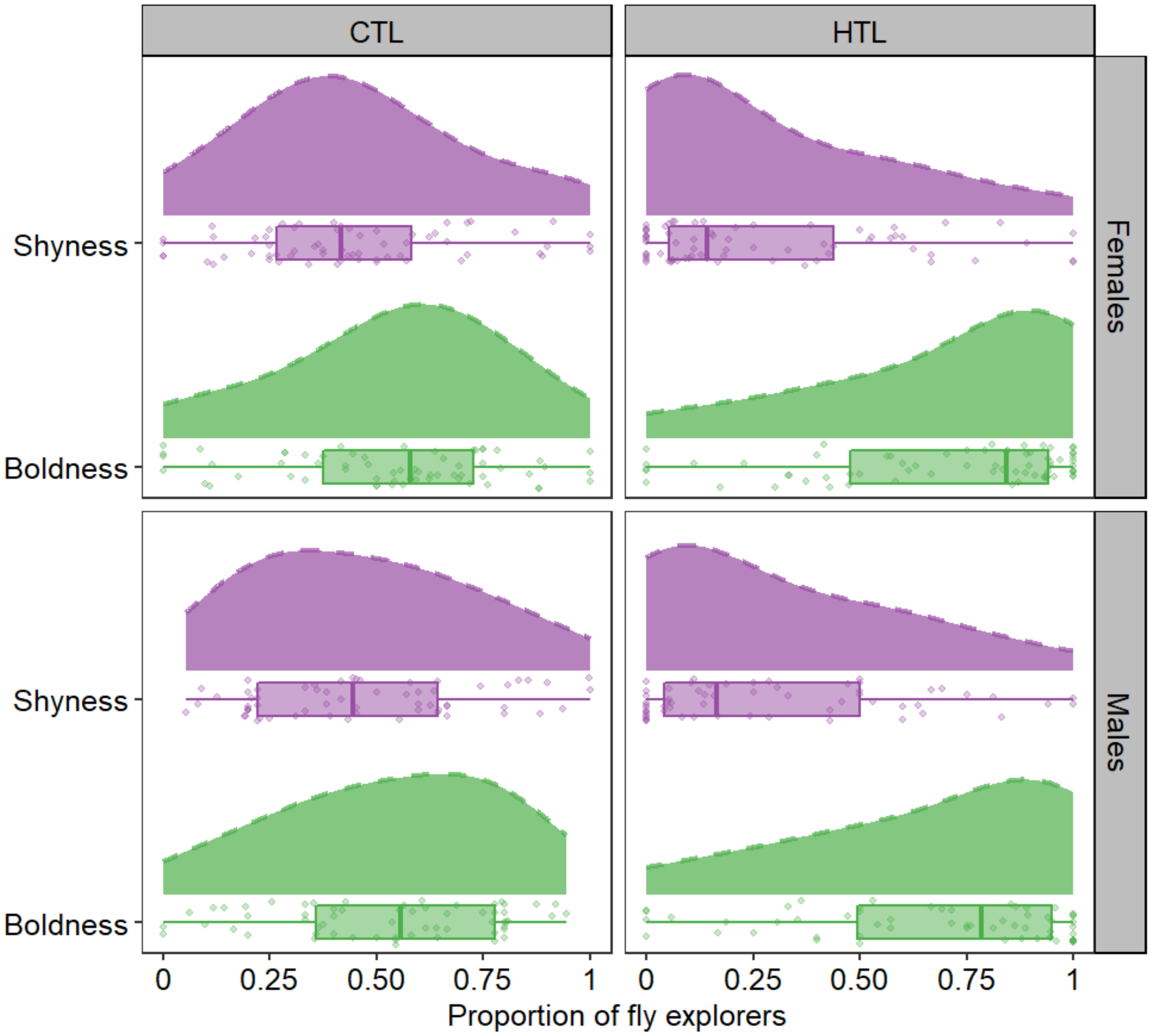


Figure S4. Association between maximum exploration vs. thermal boldness or shyness displayed by male or female fruit flies. Each data point is one fly sample. An identity line where $y = x$ is presented for visualization purposes. The Pearson's correlation coefficient (R) and p values are provided for each sex - thermal landscape combination.

Figure S4

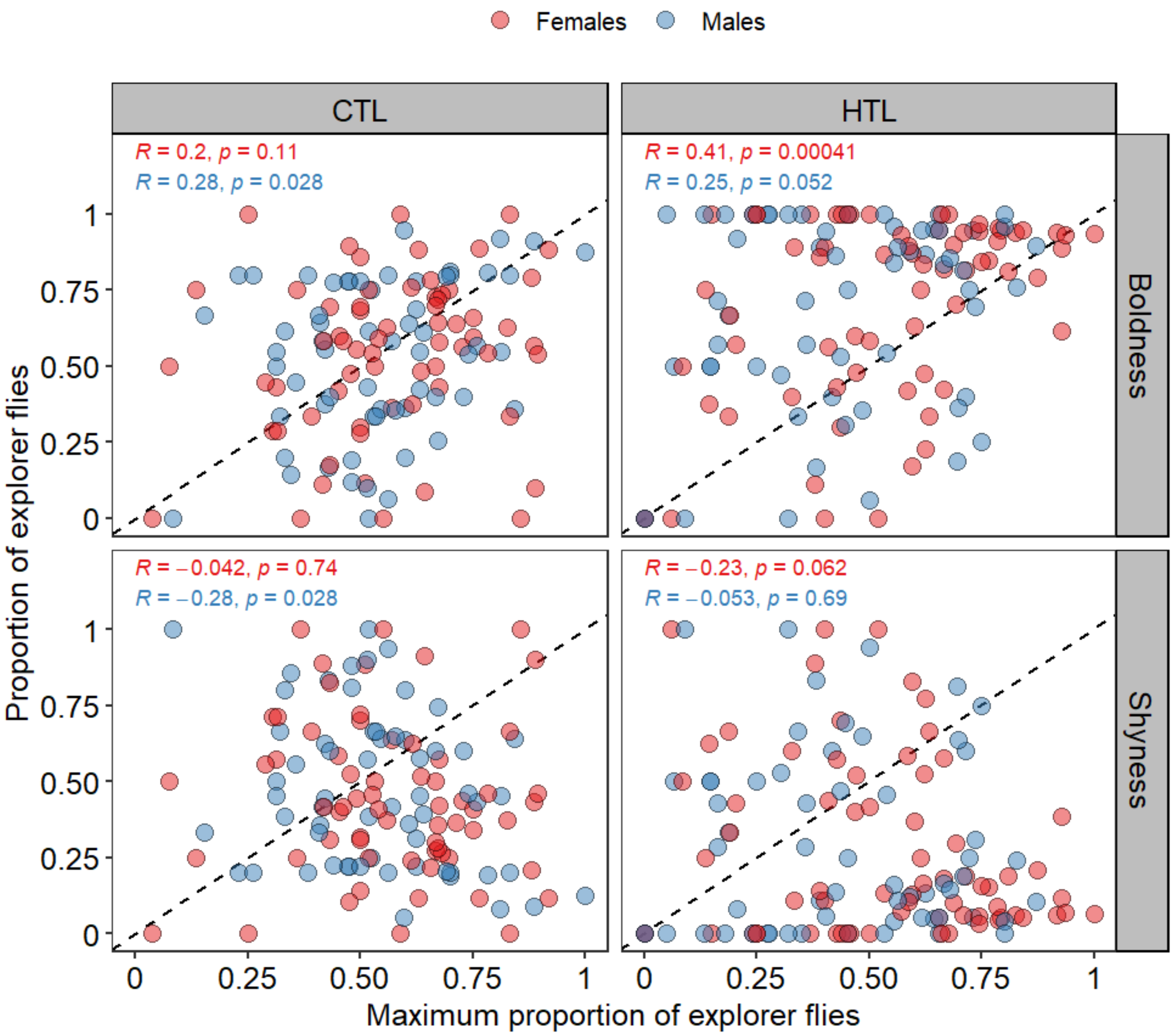


Figure S5. Association between navigation behaviors of male and female fruit flies in ETLs. Each data point is a pair of female and male samples of the same isofemale line identity that developed together (given protocol, see main text). The Pearson's correlation coefficient (*R*) and *p* values are provided for each sex - thermal landscape combination.

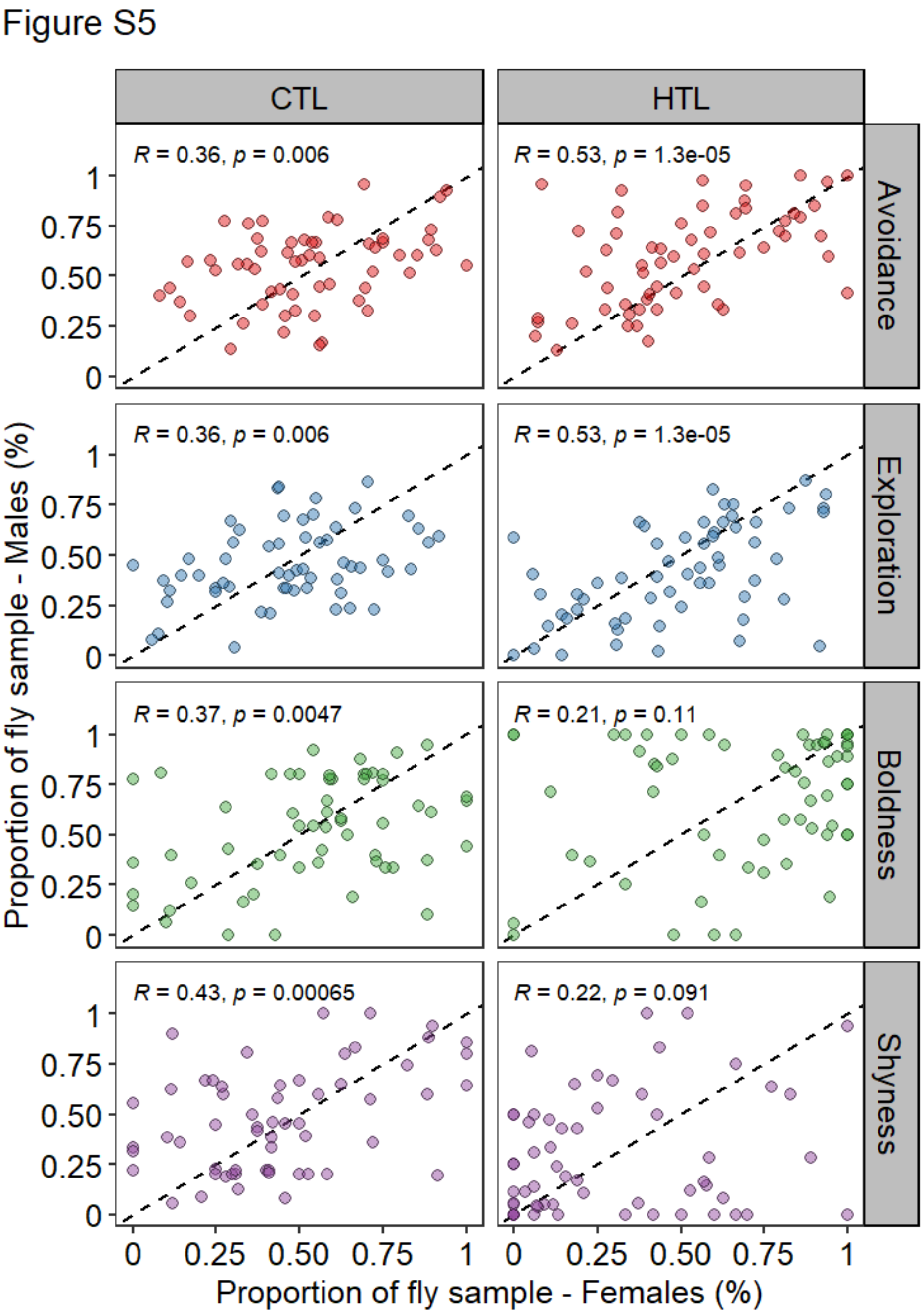


Figure S6. Among-line variation in navigation behaviors displayed by male or female fruit flies in ETLs. Bars denote the mean value for each isofemale line (among the replicates) regarding the proportion of fly sample displaying a given behavior. Error bars are ± 1 standard deviation.

Figure S6

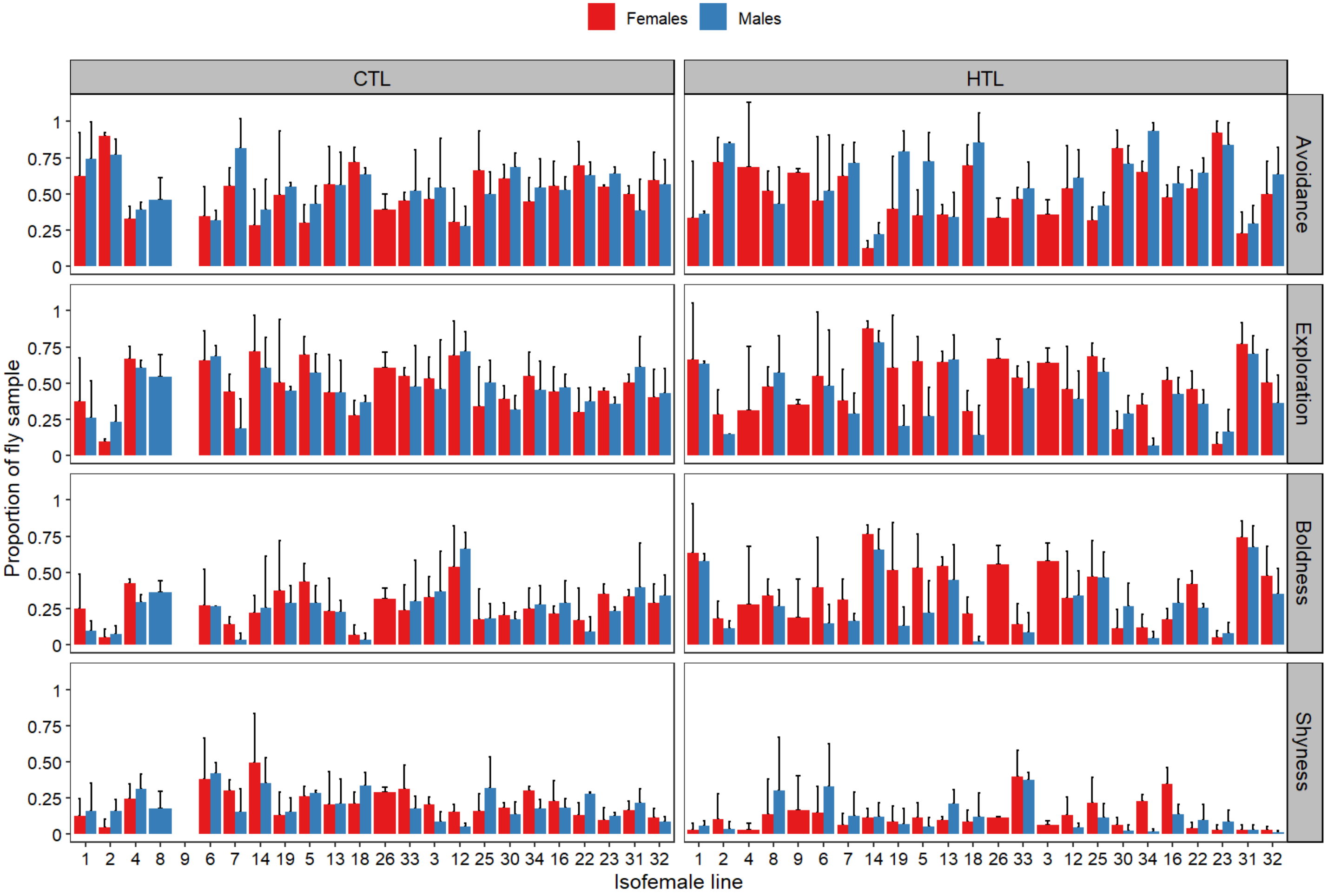
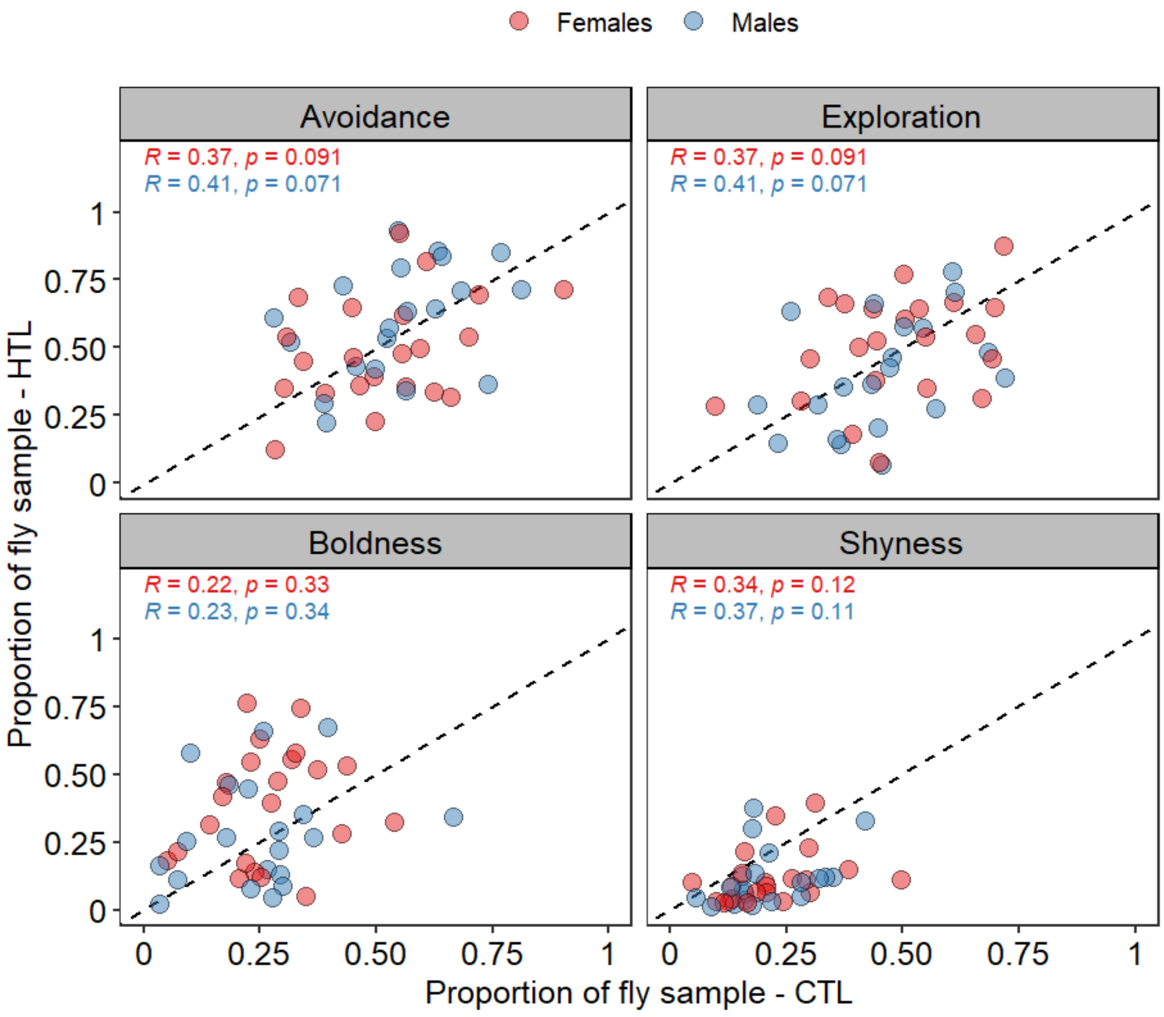


Figure S7. Phenotypic correlations (estimated as Pearson's correlation coefficients *R*) for a given navigation behavior of fruit flies between thermal landscapes, per sex. Each data point is an isofemale line (average value among replicates). An identity line where $y = x$ is given for visualization purposes. The Pearson's correlation coefficient (*R*) and *p* values are provided for each behavior-sex combination.

Figure S7



SUPPLEMENTARY VIDEOS

Video S1. Observation of fly behavior in the Cold-CTZs Thermal Landscape (CTL) early in the experiment (first 20 min). Exploration, inferred from the presence of flies in the T-System (secs 0-30), increased rapidly after the stopper is removed (Fig. 2A, Fig. 2B), but some flies also chose to stay in home bottles and do not explore the T-System (namely avoidance, secs 38-41). While few explorer flies crossed through cold CTZs (Critical Temperature Zones) and got to feeding bottles at this time (e.g., secs 16-18 and 28-30; Fig. 3A, Fig. 3B), most aggregated in the thermal gradient and kept walking calmly until the inner black rings (secs 3-14 and 24-26). Locomotor activity of flies approaching the black rings was slowed down by the cold (secs 42 through the end), and some flies entering the cold CTZs fell into Cold Behavioral Impairment [1] (see immobile fly in secs 54-58).

https://drive.google.com/file/d/1YuQQNa0HLKvxS0bVLr-CvakygdvPZuzG/view?usp=sharing

Video S2. Observations of fly behavior in the Hot-CTZs Thermal Landscape (HTL) early in the experiment (first 20 min). Exploration of the T-System also increased quickly after granting fly access (Fig. 2C, Fig. 2D). Unlike in the CTL, most explorer flies in the HTL crossed through hot CTZs as soon as they reached the thermal gradient, which concentrated thermal boldness dynamics early in the experiment (Fig. 3C, Fig. 3D). A common observation among groups was that some of the explorers that did not cross through hot CTZs stayed immobile in the middle of the thermal gradient where temperature was close to 28°C (Fig. S1B). In extreme cases, all explorer flies in an experimental group aggregated in this area, remained still, and did not cross toward any feeding bottle (secs 40-70).

https://drive.google.com/file/d/1Pmo7QxNPxumpQXUqx0I0KMbvA-tseueG/view?usp=sharing

Video S3. Four flies that crossed a hot CTZ during an HTL experiment did not enter to the adjacent feeding bottle, despite being starved. Two of these flies, observed already at sec 0, were moving slowly close to the outer black ring (≈ 36°C, Fig. S1B). A third fly was observed on its back at the space between the outer black ring and the entrance of the feeding bottle, and then it recovered its right position on sec 6. The fourth fly was at the entrance of the feeding bottle shaking the wings (secs 11-12) but not moving. Activity of these flies contrasts with activity of flies in the feeding bottle.

https://drive.google.com/file/d/13O9-KZ1HMOxuv1cGTyxUA3c4buXgDpCG/view?usp=sharing

Video S4. Exploration of the T-System with room temperature set at 19°C was largely inhibited in both thermal landscape configurations. Most flies stayed in home bottles, and yet were very active.

https://drive.google.com/file/d/1Q6bnPHoqWui5S9xcpA2UPHcOjN9qBFZg/view?usp=sharing

Video S5. One fly trying to climb through the T-System and falling in an HTL experiment conducted at 19°C room temperature, while most flies in the same sample remained in the home bottle.

https://drive.google.com/file/d/16eXJq_rdCdk7j2HYU6gpdH-ndVPjzw_j/view?usp=sharing

References

1. Navas, C. A., Agudelo-Cantero, G. A. & Loeschcke, V. Thermal boldness: Volunteer exploration of extreme temperatures in fruit flies. *Journal of Insect Physiology* **136**, 104330 (2022).

General Discussion

Climate change (CC) is predicted to become one of the most pervasive threats to biodiversity by the end of this century (CHAPIN III *et al.*, 2000; PEREIRA *et al.*, 2010; SALA *et al.*, 2000). Extreme climatic events (e.g., heatwaves) are expected to increase in frequency, duration, and magnitude under business-as-usual behaviors (FRÖLICHER; FISCHER; GRUBER, 2018; IPCC, 2021), having catastrophic consequences upon the biota, including human lives (FEYEN *et al.*, 2020; JØRGENSEN *et al.*, 2022; MITCHELL *et al.*, 2016). Reducing greenhouse gas emissions is, therefore, a compulsory and unpostponable move the ~8 billion people on Earth[10] have to make for the sake of life, including ours (COSTELLO *et al.*, 2022). Nevertheless, global warming will continue for centuries even if we stop emissions now (MATTHEWS; CALDEIRA, 2008), so mitigating and adapting to a changing climate will continue to be an utmost priority. And, in order to conduct effective evidence-based mitigation and adaptation for ourselves and the biodiversity we are part of and depend on, developing an integrative understanding about biological responses to climate remains paramount (PÖRTNER *et al.*, 2021).

This Doctoral thesis, motivated by the aforementioned problem, intends to contribute to its solution by furthering our knowledge on organismal responses to an aspect of climate: temperature. A tenet for this Doctoral investigation was that, as posed by George A. Bartholomew (1919-2006), "organism and environment form an inseparable pair", and such relationship takes place at scales relevant to body size (BARTHOLOMEW, 1964, 1966). Worth mentioning, results presented in Chapters 1-2 (*Specific objective* 1) are integrated in a larger context (Fig. 6; see

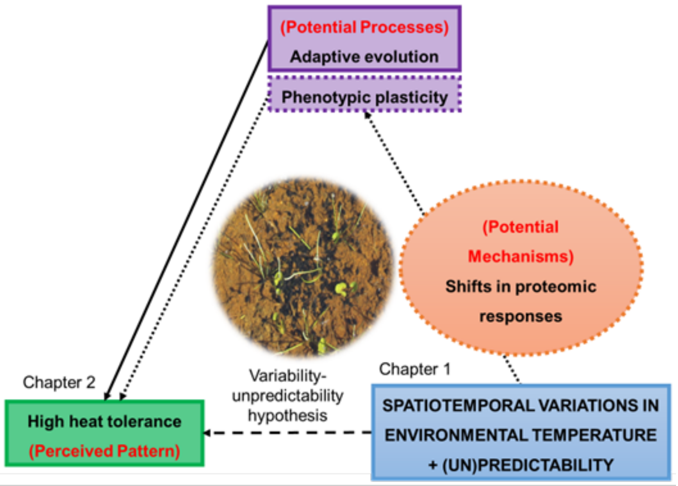


Figure 6. Potential relationships linking patterns, processes, and mechanisms under the variability-(un)predictability hypothesis proposed here (dashed-line arrow) in anuran larvae. Aspects covered in this thesis are represented by solid elements. Aspects studied under the PhD project that are still ongoing are represented by dotted elements.

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[10] https://www.un.org/en/desa/world-population-reach-8-billion-15-november-2022 Accessed on 5 December 2022.

also *General Appendix*) framed by the main research project underlying this Doctoral thesis, which sought to connect patterns, processes, and mechanisms of the relationships between thermal variations, their predictability, and the upper thermal limits (CT$_{max}$) of anuran larvae. In addition, Chapter 3 presented results of a sideline investigation conducted while I was Guest PhD Student at the Department of Biology, Aarhus University (Denmark), under the supervision of Prof. Dr. Volker Loeschcke and Prof. Dr. Jesper G. Sørensen.

We observed high microclimatic thermal diversity among aquatic microhabitats used by anuran larvae within a small patch (~1 km^2) of the Brazilian Atlantic Forest (**Chapter 1**). Main differences in thermal regimes among water bodies were related to variation in their physical characteristics (e.g., temporality, size, water flux) and associated canopy cover. Previous studies found similar results (DUARTE *et al.*, 2012; GUTIÉRREZ-PESQUERA *et al.*, 2016; PINTANEL *et al.*, 2022; SANABRIA *et al.*, 2021), though comparisons encompassed larger spatial scales than the one used here. Habitat complexity is known to drive local microclimatic diversity in terrestrial microhabitats of anurans in tropical forests, including our study location (ORTEGA CHINCHILLA, 2019; SCHEFFERS *et al.*, 2017). That a similar phenomenon occurs in aquatic microhabitats of anurans is remarkable, considering the thermal properties of water. Thus, microclimatic diversity may characterize both terrestrial and aquatic microhabitats used by anurans in tropical forests, a factor that may contribute to their high levels of alpha diversity (BASS *et al.*, 2010; PILLAY *et al.*, 2021). Specifically, microclimatic diversity sets a scenario where thermal niche divergence among anuran species with distinct natural history may be both cause and consequence of habitat partitioning (see CARILO FILHO et al., 2021), facilitating species coexistence. An example of the former, even though from a different setting, serves as illustration and is presented in Fig. 7.

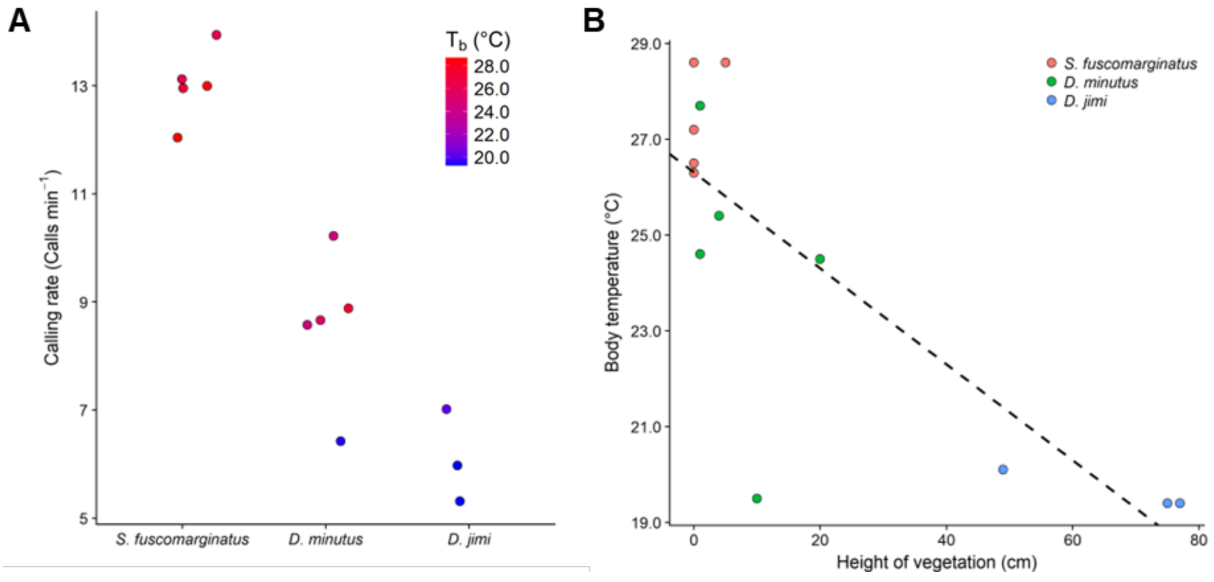

Figure 7. Microhabitat partitioning and thermal ecology of treefrogs *(Scinax fuscomarginatus, Dendropsophus minutus* and *D. jimi*) in a remnant of Cerrado, Municipality of Águas de Santa Bárbara, São Paulo State, Brazil. A) Adult males call at different frequencies over the same wetland and display body temperatures (T_b) inversely proportional to their calling activity. B) Individual T_b also relates negatively to perch height occupied. All in all, the taller on a perch, the colder the frog, and the less frequent his call is. AGUDELO-CANTERO & PADILLA-PÉREZ (Unp. data).

The anuran assemblage that inhabits our study zone harbors a high diversity of upper thermal limits at larval stages (individual CT_{max} range = 32.3 – 42.4 °C; **Chapter 2**). Interspecific physiological variation reflected more microclimatic exposure than phylogenetic relatedness, for larvae developing in similar water bodies displayed comparable CT_{max} (see below) regardless their taxonomic affiliation. Interestingly, phylogenetic independence/microhabitat association of CT_{max} has been observed for other anuran species in different regions of the Atlantic Forest (CARILO FILHO *et al.*, 2021; MADALOZZO, 2018), but not in other tropical settings (PINTANEL *et al.*, 2019, 2022; VON MAY *et al.*, 2019). In addition, intrinsic factors (body mass and ontogeny) affected little CT_{max} variation within our study anuran assemblage, but species were differentially sensitive to experimental heating rates (HRs). The latter was expected given the complex ways by which HRs affect individual physiology (REZENDE; TEJEDO; SANTOS, 2011; TERBLANCHE *et al.*, 2011), deriving on species-specific responses in both CT_{max} averages and interindividual variances to HRs (AGUDELO-CANTERO; NAVAS, 2019; CHOWN *et al.*, 2009).

As predicted, larvae of species inhabiting the warmest microhabitats (open-area ponds) were both the most heat tolerant and yet the most vulnerable to acute warming within the assemblage, because maximum pond temperatures are very close to tadpoles' CT_{max} (**Chapter 2**). Such a trend seems general for both larval and adult anurans (CHENG *et al.*, 2022; DUARTE *et al.*, 2012; GUTIÉRREZ-PESQUERA *et al.*, 2016; PINTANEL *et al.*, 2019, 2022; SIMON; RIBEIRO; NAVAS, 2015). However, since based on the *Warming Tolerance* (*WT*) metric (DEUTSCH *et al.*, 2008), diagnosis of vulnerability to acute warming in our study anuran assemblage were heavily affected by sources of CT_{max} variation (see above) and, mainly, the operationalization of T_{max}. Specifically, using both a microclimatic summary statistic far from extreme T_{max} or a macroclimatic summary statistic led to comparable and misleading conclusions about the true vulnerability of individuals and samples to acute warming, given the data. The former raises caveats on *WT*-based vulnerability assessments to acknowledge sources of variation on this metric and its extent of inference.

Fruit flies displayed diverse behavioral strategies when navigating through extremely cold or hot experimental thermal landscapes (CTL or HTL) beyond simply thermophobic responses

(**Chapter 3**). Fly behaviors seemed to be decision-based, and included the exploration or not of the experimental system, and the voluntary tendency to engage in thermal risks (namely thermal boldness; NAVAS; AGUDELO-CANTERO; LOESCHCKE, 2022), or not. The proportion of flies in a sample displaying a particular behavior varied among isofemale lines, deriving on behavioral diversity at the population level. Interestingly, both exploration of the system and thermal boldness were comparatively higher in the HTL relative to the CTL. These results countered previous observations of fly behaviors when both thermal extremes were presented together to flies (NAVAS; AGUDELO-CANTERO; LOESCHCKE, 2022), suggesting that flies modulate their behaviors based on the thermal landscape configuration. Moreover, fly behaviors displayed higher (total) genetic variance among isofemale lines, broad-sense heritability, and evolvability in the HTL than in the CTL, and were not genetically correlated between thermal landscapes. These patterns oppose well-established evidence on the thermal physiology of *D. melanogaster* showing stronger constraints on the evolution of heat vs cold tolerance (HOFFMANN, 2010; HOFFMANN; CHOWN; CLUSELLA-TRULLAS, 2013; KELLERMANN *et al.*, 2012). Then, thermal physiology and behavior may respond in different, and yet always intertwined ways to extreme temperatures. At this respect, a common view is that thermoregulatory behaviors constrain the evolution of thermal physiology in motile forms (the "Bogert effect"; BOGERT, 1949), because the combination of thermophobic responses to thermal extreme and "the seek" for optimal temperatures to function shield organisms from selection on their thermal limits (HUEY; HERTZ; SINERVO, 2003; MUÑOZ, 2022). However, behavioral diversity, and especially thermal boldness, calls back to the most classic view in evolutionary theory where behavior may drive (physiological) evolution and range expansions (HUEY; HERTZ; SINERVO, 2003; MAYR, 1960, 1963).

In conclusion, we confirmed that spatial and temporal thermal variations at scales relevant to organisms favor physiological and behavioral diversity across species (as was the case for anuran larvae) and within populations (as seen in fruit flies). Results presented in this Doctoral thesis can be useful to develop mechanistic forecasts of organismal responses to climate warming. This thesis draws attention that a better comprehension of microclimates *per se*, particularly spatial heterogeneity (WOODS; DILLON; PINCEBOURDE, 2015) and temporal predictability (REED *et al.*, 2010), is essential to understand both impacts of climate warming on organisms and their responses (NADEAU; GIACOMAZZO; URBAN, 2022). Understanding and protecting the inherent diversity of life is essential to safeguard its future in the Anthropocene (CORLETT, 2020; LEWIN *et al.*, 2018; WILSON, 2002).

General Conclusions

1. Within the same habitat, water bodies used by anuran larvae filter differently the local atmosphere given differences in their physical structure (temporality, size, water flux) and associated canopy cover, which leads to differences in their temporal thermal variation over very short distances (meters, **Chapter 1**).

2. The microclimatic thermal diversity that we report here at a local scale (~1 km^2) is comparable with microclimatic variation previously observed across macroecological clines (e.g., latitude or elevation), and it is not detected by coarse-gridded climatic descriptors (e.g., weather stations; **Chapter 1**).

3. Paralleling local patterns of microclimatic diversity, our study anuran assemblage displays high diversity of upper thermal limits (CT_{max}) at larval stages. This result contradicts Brett's rule, in the sense that CT_{max} can vary greatly among species and at microgeogaphic scales (**Chapter 2**).

4. Although we compared a small set of species, our phylogenetic considerations indicate that the CT_{max} of our study species is little related to phylogenetic relatedness and more to microhabitat use, for species sharing similar water bodies had comparable CT_{max} regardless despite their phylogenetic distance (**Chapter 2**).

5. As predicted by the Climate Variability Hypothesis, the most heat tolerant species inhabit the warmest and more thermally variable microhabitats (ponds in open areas), whereas the less heat tolerant species were restricted to cooler and more thermally constant water bodies inside the forest. Paradoxically, the most heat tolerant species are also the most vulnerable to acute warming, since they are living close to their upper thermal limits (**Chapter 2**).

7. Body mass and ontogeny contributed little to CT_{max} variation across species and within a target species, respectively. Yet, it is premature to generalize the effects of these factors on the heat tolerance of anurans. In the case of ontogeny, current evidence suggest that our results are exceptional. Given amphibian natural history,

assessments of vulnerability to warming should consider body-size and ontogenetic-related variation in thermal traits (**Chapter 2**).

8 The *WT* metric, and inference thereof, are sensitive to variation in their components, namely CT_{max} and T_{max}. Acknowledging relevant sources of CT_{max} variation and proper operationalization of T_{max} are critical aspects for *WT*-based vulnerability assessments (**Chapter 2**).

9 Canopy is a critical factor modulating both microclimatic heterogeneity and the vulnerability of anuran larvae to warming. Protecting and restoring the canopy is critical to protect the anuran fauna of tropical forests (**Chapter 1, 2**).

10 Fruit flies display diverse and complex behavioral responses beyond thermophobia while navigating through extreme thermal landscapes. Moreover, flies modulate their behaviors in response to the configuration of the thermal landscape (**Chapter 3**).

11 Navigation behaviors of flies displayed higher genetic variance, broad-sense heritability, and evolutionary potential in extremely hot rather than cold thermal landscapes, as opposed to comparable evidence for thermal limits. The integration of physiology and behavior to respond to spatiotemporal thermal variations in the wild call attention to continue testing both the Bogert effect and alternative evolutionary hypothesis of thermal adaptation (**Chapter 3**).

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General Appendix

This Doctoral thesis was based on an integrative approach designed to link (1) *patterns* of environmental and physiological variation with potential (2) *processes* producing those patterns and (3) *mechanisms* underlying organismal responses to temperature variability and predictability. Consequently, such an approach was interdisciplinary and involved multiple efforts, some still ongoing by the time this thesis was to be submitted (see Fig. A1 at the end of this section). One such effort relates to understand whether anuran larvae can modify their CT_{max} in response to different scenarios of thermal variation and predictability experienced during development. We used the American bullfrog *Lithobates catesbeianus* (SHAW, 1802) as model organism for this test. Three egg clutches (different dams, ~ 1000 eggs each) were acquired from a frog farm 24 h after spawning, and separated into three independent aquariums each ($N = 9$; Fig. 3A). Water temperature was set at 25 ± 1°C until embryos hatched into free-swimming tadpoles (~ 5 days). Then, the thermal treatments were activated by means of aquarium heaters and timers (the latter for setting the variable thermal regimes). A glance into the experimental setup and thermal regimes is provided in Figure A2.

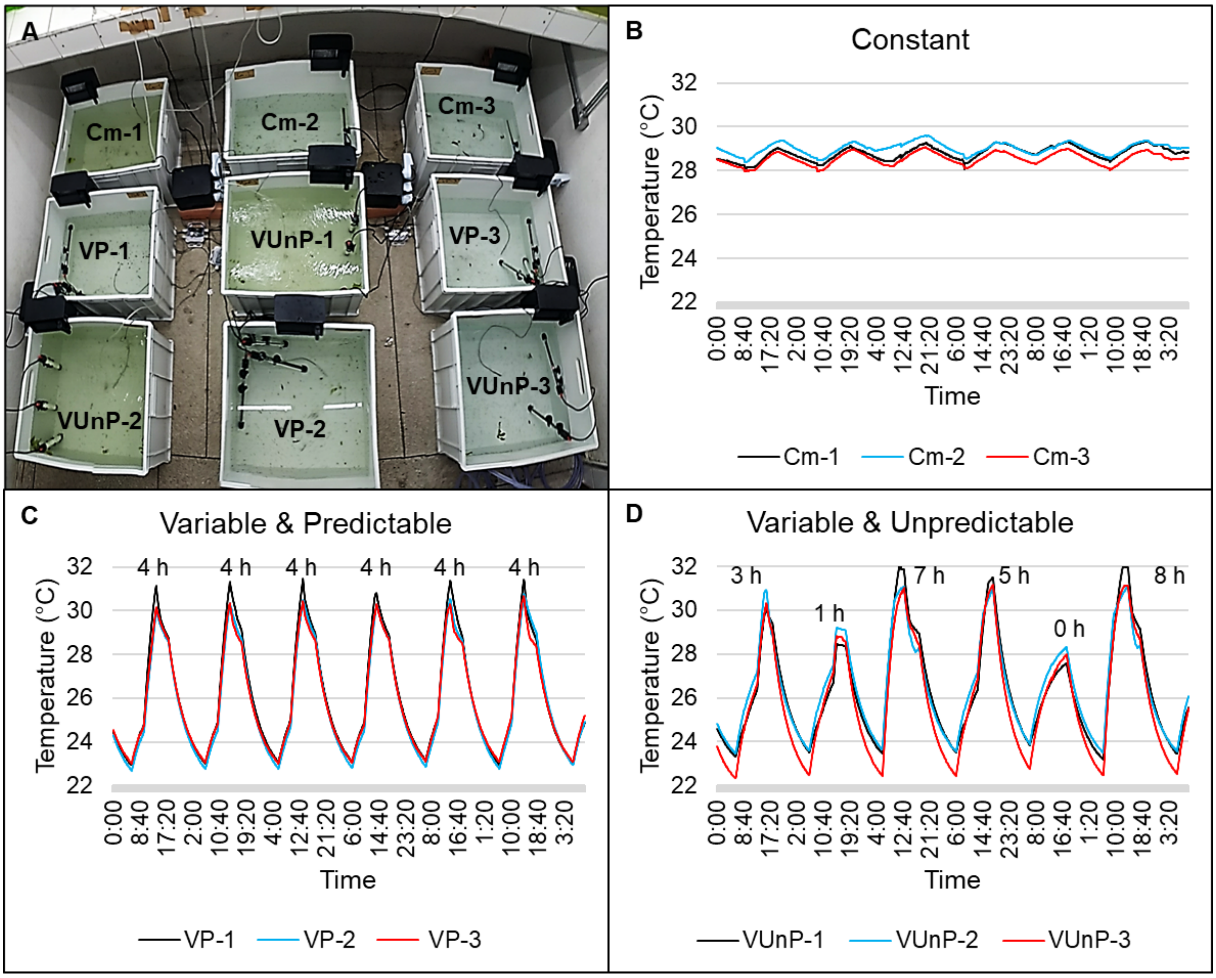

Figure A2. Experimental setup and thermal regimes (6 days) used in this study. A) Independent aquariums and distribution of thermal regimes. Each egg clutch was distributed into one level of each treatment. B) Constant and moderate (Cm) thermal regime with mean temperature set at 28 ± 1°C. C) Variable and predictable (VP) thermal regime (23-33°C), with 4 h of diurnal heating every day at the same time. D) Variable and unpredictable (VUnP) thermal regime, with 0-8 h of diurnal heating varying stochastically among days.

After 21 days, three samples of tadpoles ($N = 18$) were collected from aquariums, of which two were used for CT_{max} experiments at an ecologically relevant HR (0.05°C/min), and the remaining was used as control (i.e., was manipulated as in a CT_{max} experiment but did not undergo heating). Six hours after CT_{max} experiments, five tadpoles per group were flash frozen in liquid nitrogen for whole-organism proteomic analyses. With these data, we shall characterize the within-family thermal reaction norms (ANGILLETTA, 2009) of CT_{max} and link them with their underlying mechanisms at the proteomic level.

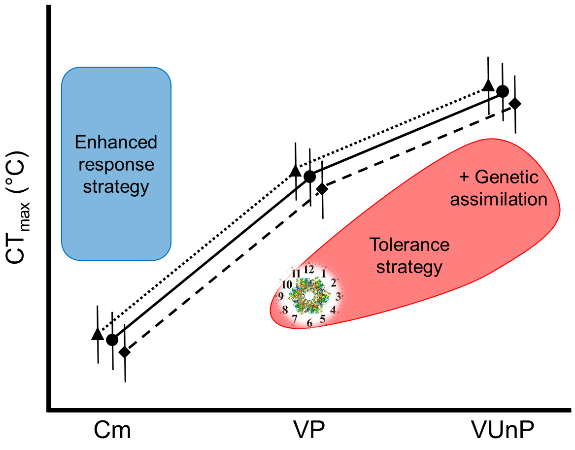

Figure A3. Predicted within-family thermal reaction norms for the CT_{max} of *L. catesbeianus* tadpoles in response to three different simulated scenarios of temperature variation and predictability. Shapes depict mean CT_{max} of the different egg clutches, whereas lines connecting same shapes indicate within-family acclimation scope (mean CT_{max} differences). Predicted molecular strategies underlying CT_{max} response are presented according to the main text.

According to our variability-(un)predictability hypothesis, we shall test the following predictions (Fig. A3):

a) At the organismal level, CT_{max} will be higher in tadpoles acclimated to the VUnP, intermediate in those acclimated to the VP, and lower in siblings acclimated to the Cm.

b) The within-family acclimation scope of CT_{max}, a measure of its plasticity, will be higher between the Cm and the VP thermal regimes, and lower between the VP and the VUnP.

c) An enhanced response strategy will characterize the proteome underlying CT_{max} responses to the constant thermal regime, whereas a tolerance strategy will typify molecular pathways responding to the variable thermal regimes.

d) While the predictable environment may elicit circadian controls, the unpredictable environment may induce a stronger response (more proteins) and higher constitutive levels of mechanisms.

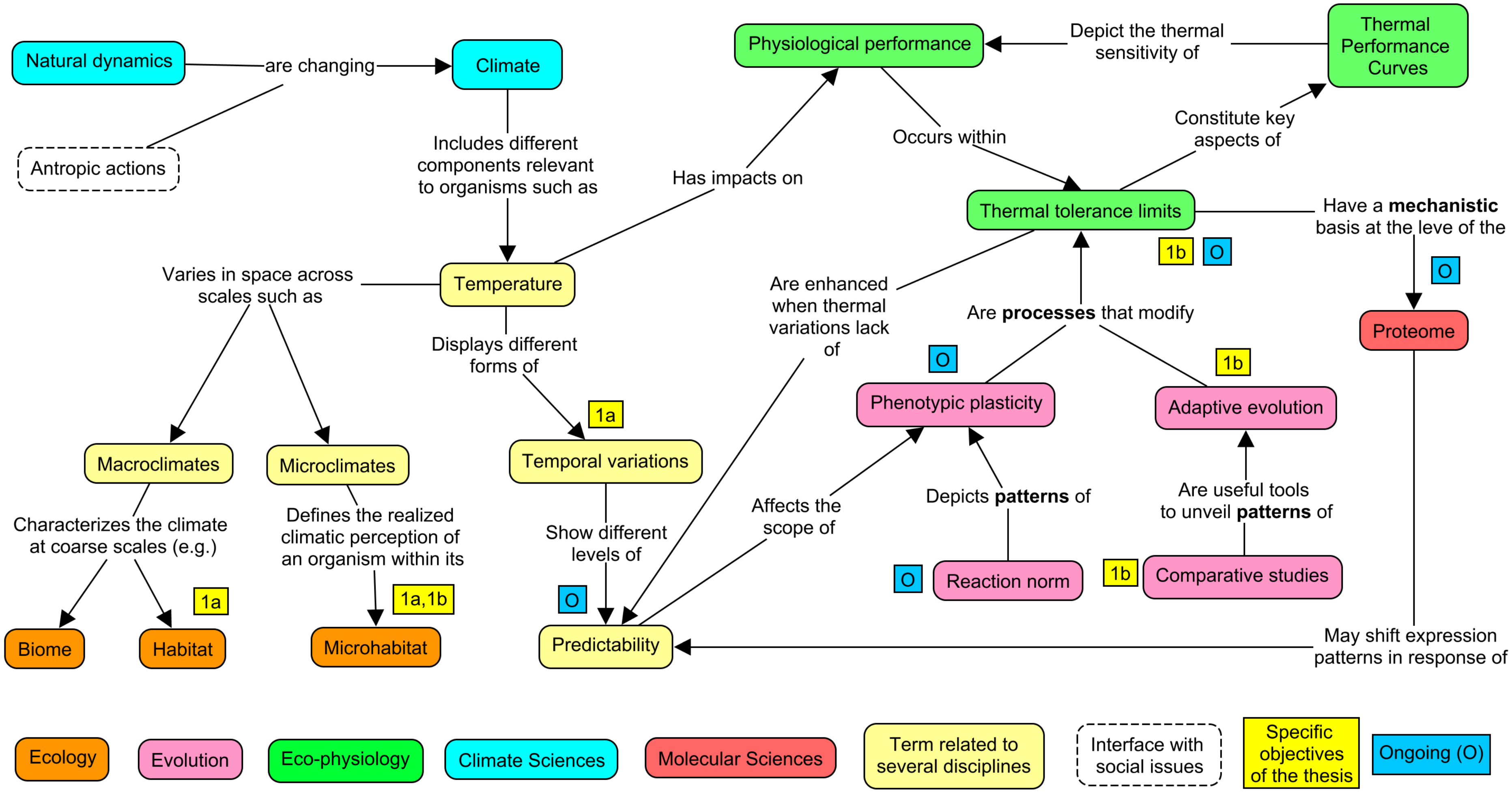

Figure A1. Concept map integrating the interdisciplinary theoretical framework and of the main Doctoral research project. Insertion of specific objectives tackled in this thesis, as well as ongoing studies, are shown.