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Butterfly thermoregulation across habitats and climates

Ph.D. Thesis

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Annotation

Global warming, through rising temperatures and changing precipitation patterns, is placing major stress on species and ecosystems. Understanding how species respond to temperature and the mechanisms underpinning thermoregulation can help us predict which species are most vulnerable in the face of warming. In this thesis, I explore how butterflies across different habitats and climates thermoregulate and the mechanisms, including morphology and behaviour, underlaying thermoregulatory ability. Firstly, when comparing the buffering ability of neotropical and temperate butterflies I found that tropical butterflies were able to maintain more stable body temperatures than temperate butterflies, and this was likely driven by their morphology. I also found that temperate butterflies used postural means to raise their body temperature more than neotropical species, likely an adaptation to the cooler air temperatures they experience. Secondly, I showed the importance of butterflies' thermoregulatory abilities at the community level, by comparing thermoregulation of European butterflies across geographic regions and climatic zones. This study highlighted that behavioural thermoregulation, including the use of microclimates and postural means, drives regional differences in butterflies' thermoregulatory abilities. Finally, I utilised the Müllerian mimicry exhibited in *Heliconius* butterflies to untangle the contributions of morphology and phylogeny in butterfly thermoregulation, investigating thermal traits, including buffering ability, take-off temperature and heating rate. I found that morphology, not phylogeny, was the main driver of thermoregulation in these butterflies. Further, I investigated differences in the thermoregulatory ability of *Heliconius* butterflies from different habitats. I found that species from colder habitats were able to maintain a more stable body temperature and took off at a lower temperature than those from hotter habitats, suggesting there is local adaptation or acclimation in thermal traits. Overall, this work highlights that species have their own unique thermoregulatory abilities, as a result of the thermal environment they experience, and that thermoregulation is driven by morphology, behaviour and physiology. My findings have important consequences for predicting the impacts of climate change on ectotherms, by highlighting variation in thermal ability which makes some populations and species more vulnerable, while others more resilient. This thesis lays the groundwork for

future studies comparing species' thermal traits across climates and habitats, increasing our understanding of how species cope with climate and land-use change.

Declaration

I hereby declare that this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared and specified in the text.

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Katerina Sam, the supervisor of this Ph.D. thesis and co-author of all of the manuscripts included in this thesis, fully acknowledges the major contribution of Benita C. Laird-Hopkins to these manuscripts.

Mgr. Katerina Sam, Ph.D.

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Chapter 1

Introduction

Climate change is resulting in an increase in global temperatures and heatwave events as well as changes in precipitation patterns (Lee et al., 2021). Global temperatures have risen on average 0.85°C from 1880 to 2012 and are predicted to continue to rise (Lee et al., 2021). Rainfall patterns have been altered with an increase in extreme precipitation events and a reduction in mean annual precipitation in many parts of the world (Imbach et al., 2018; Lee et al., 2021). This directly impacts species resulting in changes in their physiology, morphology, life history and distribution (Angilletta, 2009; Franco et al., 2006; Parmesan, 2006; Sunday et al., 2014). Indirectly it causes changes in the distribution and abundance of predators, parasites and hosts (Diamond et al., 2011; Parmesan, 2006). Considering this, the effects of changes in temperature and humidity on species are wide-reaching, with consequences being felt across entire ecosystems as species effects cascade throughout trophic levels (Eggleton, 2020; Parmesan, 2006; Sunday et al., 2014). Consequently, gaining an insight into how species respond to changes in temperature and humidity is pivotal to predict the impacts of climate change.

To determine how climate change will impact species it is important to develop our understanding of the traits which dictate species sensitivity to temperature (Diamond et al., 2011; Diamond & Yilmaz, 2018). These include morphological, behavioural and physiological traits and can be the consequence of the climatic conditions a species experiences (Bonebrake et al., 2014; González-Tokman et al., 2020; Wenda et al., 2021; Xing et al., 2016). Major differences in temperature and humidity are found between climates and habitat types (Lee et al., 2021; Metzger et al., 2013; Montejo-Kovacevich et al., 2020). Across latitudes, tropical regions exhibit a narrower temperature range and higher mean annual temperature than temperate regions (Lee et al., 2021). Within latitudes, different bioclimatic regions have very different climatic conditions. This is most noticeable across an altitudinal gradient, whereby lowland regions have higher temperatures than highland regions (Metzger et al., 2013). Temperature and humidity availability differs between habitat types, for example the forest

understory buffers extreme temperatures, resulting in lower and more stable temperatures as well as higher humidity levels compared to more open habitats (De Frenne et al., 2021). Even within habitats temperature and humidity levels vary greatly, whereby distinct microclimates occur across a narrow spatial range (Montejo-Kovacevich et al., 2020; Pincebourde & Suppo, 2016). Such microclimates can magnify as well and buffer the consequences of increasing temperatures (De Frenne et al., 2021; Pincebourde & Suppo, 2016; Pincebourde & Woods, 2020). Across all these scales temperature variation plays a pivotal role in species' thermal adaptation. It remains unclear how increasing temperatures at the macroclimate scale impact temperatures at finer scales, and how this influences species' responses to climate change (Jucker et al., 2020). In this thesis, by combining in situ measurements and laboratory experiments, I study at the global, regional, habitat and microclimate scale how butterfly thermoregulation is driven by morphology and behaviour and to what extent individuals are adapted or acclimatised to the specific environment they inhabit.

1.1 Butterflies as a study system

Butterflies perform a number of crucial ecosystem services and are the foundation of many ecosystems (Bonebrake et al., 2010). The larvae are an important food source and may contribute to the huge species diversity found in the tropics, and the adults play a pivotal role as pollinators (Lewinsohn & Roslin, 2008). Butterflies are thought to have diversified about 100-60 million years ago, with diversity patterns partially explained by large scale climatic changes including glacial retreat (Megens et al., 2004; Wahlberg et al., 2009). Globally there are approximately 18,000 – 20,000 species of butterflies, with 90% of this diversity found in tropical regions (Bonebrake et al., 2010). In both tropical and temperate regions butterflies exhibit strong seasonality patterns, which are often triggered by photoperiod, climatic conditions, including temperature and humidity, and/or the availability of host plants (Colom et al., 2022; Jones & Rienks, 1987). In temperate regions butterflies undergo diapause in the winter when temperatures are low (Lehmann et al., 2018). In tropical regions diapause is often undergone in the dry season when water availability is limited (Srygley et al., 2014). Migratory butterflies, which can travel thousands of miles, such as *Danaus plexippus*, avoid the cold winter temperatures of higher latitudes by relocating to warmer regions, such as Mexico

(Masters et al., 1988). As a result of these life history strategies there is strong seasonality in butterfly populations in both tropical and temperate regions (Grøtan et al., 2014). In addition to seasonal changes in butterfly populations, butterfly populations differ markedly between habitat types and microclimates as a consequence of host plant availability and climatic conditions (Beirão et al., 2021; Checa et al., 2014; Cómbita et al., 2022). A butterflies physiological functions are influenced by temperature and humidity and so they are particularly vulnerable to changing environmental conditions (Deutsch et al., 2008). Consequently, climate change, in combination with widespread pesticide use and a reduction in suitable habitats, is causing a decline in butterfly populations (Eggleton, 2020). It is difficult to predict their vulnerability to future climate change as little is known about their thermoregulatory abilities, especially of butterflies from tropical regions where the largest proportion of their abundance and diversity is found (Bonebrake et al., 2010; Gaston, 2000). Understanding how they respond to climatic conditions is crucial for determining the ecosystem-wide impacts of climate change (Bonebrake et al., 2010; Harvey et al., 2020).

One genus of butterflies, *Heliconius*, has long been the interest of Lepidopterists. They exhibit Müllerian mimicry, in which distantly related but co-occurring species share the same colour pattern (Jiggins, 2017). The adult's colouration is aposematic, a way to advertise their toxicity to predators, as a consequence of the cyanogenic compounds the larvae sequesters from its host plants, *Passifloraceae*. They are the only butterflies known to feed on pollen, providing them a source of protein which increases their longevity and brain size (Montgomery & Merrill, 2017). Consequently, they are able to memorise the location of nectar plants and in doing so follow traplines (Young & Montgomery, 2020). *Heliconius* are found throughout the Neotropics and to date there are 48 described species. Genetic work has helped identifying the species, determining which individuals are mimetics within the genus and which mimetics belong to other genera. They exhibit distinct niche partitioning with some species present only in forest interiors and others preferring more open habitats (Estrada & Jiggins, 2002). Despite dispersing relatively large distances after emergence, 2-4 km, as adults they establish small home ranges, in an effort to memorise and navigate their environment, indicating that there are high levels of local adaptation (Ehrlich & Gilbert, 1973; Mallet, 1995). Local adaptation to high altitude has been shown in *H. erato* and *H. melpomene*, suggesting adaptation to different habitats might also occur in other *Heliconius* species (Montejo-

Kovacevich et al., 2022). The extensive knowledge of their natural history and the origins of their colour patterning as well as the high levels of niche partitioning observed in these species, makes them the ideal system for studying the drivers of thermoregulation.

1.2 Variation in climate sensitivity across habitats and climates

Butterflies from different habitats and climates may differ in their sensitivity to temperature and rainfall (Melero et al., 2022; Montejo-Kovacevich et al., 2022). Tropical species are thought to be more vulnerable to temperature changes than temperate species, as they experience a narrower range of temperatures and so are less able to cope with variability (Janzen, 1967). Additionally, they live closer to their thermal maximum and so may be more at risk to similar levels of warming (Deutsch et al., 2008; J. Sunday et al., 2019). However, recent studies suggest that certain temperate species might be just as vulnerable to climate change, especially temperate species which have a short activity period and hence only experience a narrow range of temperatures (Colado et al., 2022; Johansson et al., 2020; Mi et al., 2022). In addition to temperature, precipitation drives butterfly population trends, with species from arid regions decreasing most in abundance compared to humid habitats (Herrando et al., 2019). Across altitude butterflies' thermal tolerance varies, with low altitude populations having a higher heat tolerance than high altitude butterflies (Fischer & Karl, 2010; Montejo-Kovacevich et al., 2020). Additionally, high altitude butterflies start flying at lower body temperatures than those from low altitudes, likely an adaptation to the colder air temperatures found at high altitudes (Advani et al., 2019; MacLean et al., 2016). Differences in thermoregulatory ability are also observed between populations and species from different habitats, with those from spatially heterogeneous environments having a greater thermal tolerance than those from homogeneous habitats (Bonebrake & Deutsch, 2012; Dongmo et al., 2021; Kleckova et al., 2014). Ecotone butterfly populations, which experience high temperatures, have a higher thermal tolerance than those living in forest interiors where temperatures are lower (Dongmo et al., 2021). Similarly, grassland species are adapted or acclimatised to warm meadows, being active at higher air temperatures compared to forest dwelling species (Kleckova et al., 2014). Thermal tolerance and drought resistance also varies across microclimates, with ants living in drier microclimates, such as the forest canopy, being more drought resistant than those living in the humid forest understory (Bujan et al., 2016).

We lack, especially from tropical regions, systematic field studies investigating the thermoregulatory abilities of species across climates and habitats. Through understanding how butterflies from different environments respond to temperature at the microclimate scale we can begin to predict species specific impacts of changes in climate and land-use.

1.3 The importance of morphological and behavioural traits

Butterflies use behavioural, morphological and physiological means to regulate their temperature (Bladon et al., 2020; Kearney et al., 2009; Kemp & Krockenberger, 2002; Wenda et al., 2021; Xing et al., 2016). They undertake specific behaviours, such as orientating their wings relative to the sun, either to aid with heating up or cooling down (Kemp & Krockenberger, 2002; Shanks et al., 2015). They also select favourable microclimates, to avoid heat stress when air temperatures are high or to warm up when air temperatures are low (Kleckova et al., 2014; Sunday et al., 2014). This is particularly beneficial in lowland tropical forests, where microclimate temperatures can be 2°C cooler than ambient temperatures, which at midday are frequently close to an individual's thermal maximum (Deutsch et al., 2008; Montejo-Kovacevich et al., 2020). Butterflies also migrate to cooler climates, either to higher elevations or latitudes where temperatures are colder.

Morphological traits, including wing colour, reflectance and size are all important in determining the thermoregulatory ability of butterflies (Bladon et al., 2020; Krishna et al., 2020; Shanks et al., 2015; Tsai et al., 2020; Xing et al., 2016). Light coloured butterflies actively thermoregulate by reflecting solar radiation onto their thorax to warm up or away from it to avoid heat stress (Shanks et al., 2015). Dark coloured butterflies heat up more quickly than light coloured butterflies and are found more commonly in cool habitats, such as forest interiors or at higher latitudes, where being able to heat up rapidly increases fitness (Günter et al., 2019; Xing et al., 2016). Hence, as temperatures increase with climate change it is thought light coloured insects will be selected for (Zeuss et al., 2014). Iridescence species are able to maintain lower body temperatures than non-iridescence species, as the high visible reflectance and inferred emittance properties of their wings aid in radiative cooling (Krishna et al., 2020; Tang et al., 2022; Tsai et al., 2020; Wilts et al., 2017). Big butterflies are more effective at thermoregulating than small butterflies as they may leverage their larger wing

area to either reflect solar radiation onto their thorax or away from their thorax (Bladon et al., 2020; Wenda et al., 2021). Furthermore, size is related to dispersal ability, and so larger butterflies may move to and utilise preferential microclimates.

1.4 Quantifying thermoregulation

There are a number of metrics that are measured to determine the thermoregulatory abilities of ectotherms. These include, upper critical maximum, the temperature at which an individual loses motor control (Gilbert & Miles, 2017). Thermal performance curves, the fitness of an individual across a specific temperature regime, is also measured (Sinclair et al., 2016). Similarly, survival curves, whether an individual survives after experiencing a specific temperature regime, are commonly determined. Recent studies have undertaken measurements of thermal buffering ability, whereby the individuals body temperature is measured in situ along a range of air temperatures and a slope of the regression between body temperature and air temperature provides an indication of the individuals buffering ability (Bladon et al., 2020; Kleckova et al., 2014). A shallow slope would indicate a good ability to buffer, as body temperature remains relatively stable over a wide range of air temperatures, whereas a steep slope would indicate a bad buffering ability, as body temperature changes over a wide range of air temperatures. Butterflies loose heat through convective cooling during flight, and so before take-off they must gain sufficient heat to extend their time flying (Advani et al., 2019). Flight is an important function of fitness as it allows for the search of mates, food resources and oviposition sites as well as aids in the avoidance of predators (Kemp & Krockenberger, 2002; Kingsolver & Watt, 1983). The rate at which an individual heats up (heating rate) before flight, as well as the temperature at which an individual can take-off (take-off temperature) therefore have strong fitness consequences. Heating rate in live and dead butterflies provides insight into how behavioural and physiological processes influence thermoregulation and the extent to which morphology is important (Bonebrake et al., 2014; Tsai et al., 2020; Xing et al., 2016). Undertaking ecologically relevant trait measurements, such as measurements of buffering ability, heating rate and take-off temperature, aids in understanding how these species respond to temperature changes in their natural environment. Additionally, undertaking measurements in situ elicits an understanding of how behaviour and morphology determine thermoregulation. Through studying in an ecological

context how butterflies, when sedentary as well as in flight, thermoregulate we gain an understanding of their thermoregulatory abilities throughout their activity period. We lack ecologically relevant thermoregulatory measurements for many species, which hinders accurate predictions of species response to warming and hence conservation efforts.

1.5 Outline of thesis

In this thesis, I investigate thermoregulation in butterflies from the global to the microclimate scale (Fig. 1.1). In **Chapter 2**, firstly I study butterfly thermoregulatory ability at the global scale, by measuring *in situ* buffering ability of neotropical and temperate butterflies and making comparisons at the assemblage and family level. Secondly, I determine how morphological and behavioural traits effect thermoregulation and investigate any differences in these traits between tropical and temperate species. In **Chapter 3**, firstly I measure in the field and describe the thermal buffering ability of temperate butterflies from across bioclimatic regions in Europe and investigate how they use microclimates and behaviour for thermoregulation. Secondly, I investigate the extent to which regional differences in these butterflies' buffering ability is driven by geography or climate. Thirdly, I assess if geography and climate can explain any intraspecific variation in the use of behaviour for thermoregulation in these butterflies. In **Chapter 4**, I study the thermal traits of *Heliconius* butterflies from different habitats by undertaking *in situ* measurements and laboratory experiments of wild and insectary reared populations. Firstly, I determine to what extent morphology and phylogenetic relatedness drive thermoregulation in these species. Secondly, I assess how *Heliconius* butterflies from different habitats differ in their thermoregulatory abilities, comparing species from a range of habitats, including high altitude habitats, closed canopy forests, forest edges and gaps, and mixed habitats. Finally in the **Summary**, I discuss the impact, challenges, and future directions of the work I have presented in this thesis.

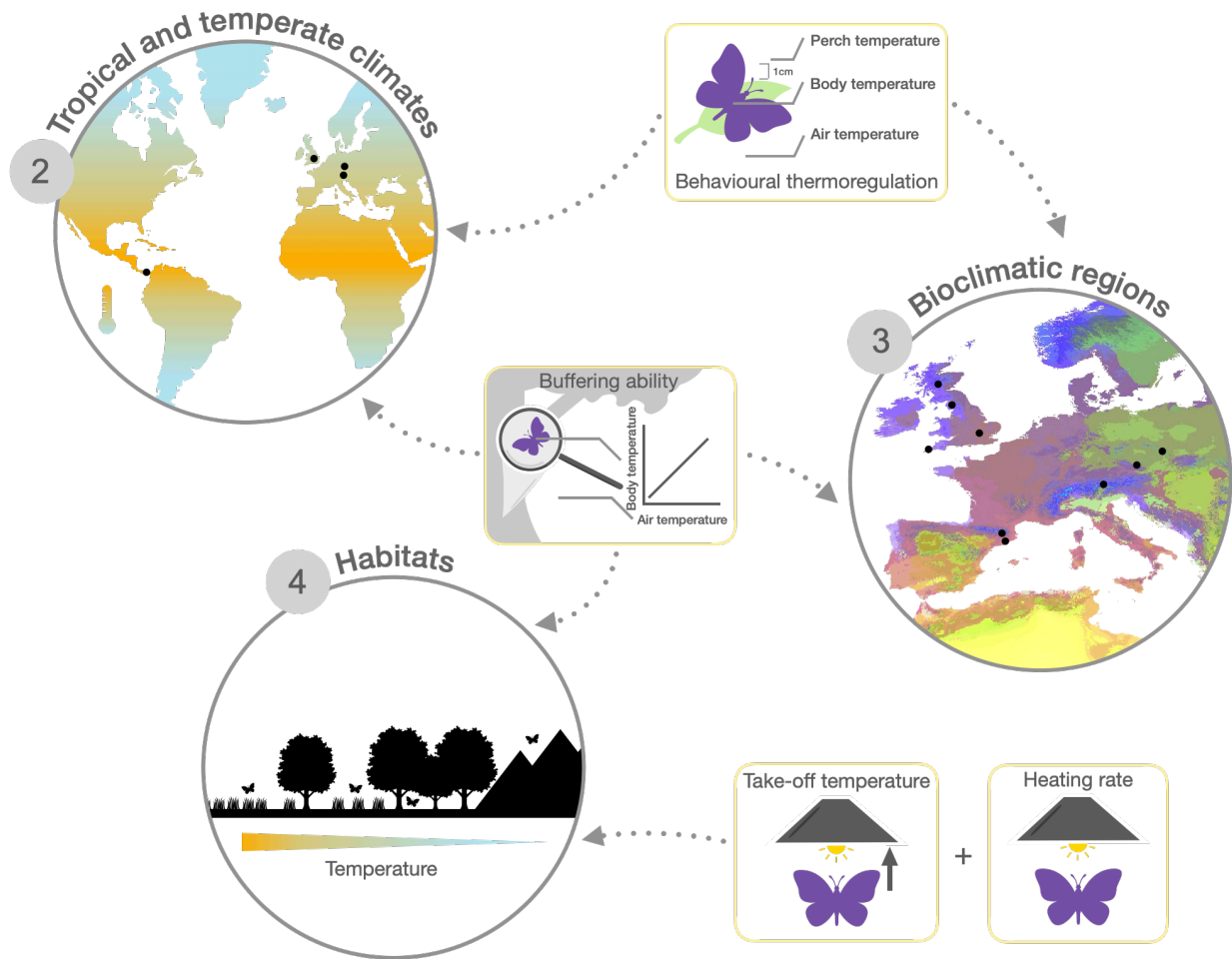


FIGURE 1.1. Graphical thesis outline. Numbers indicate the corresponding chapters, yellow boxes show the methods, and dotted arrows indicate which methods were used in the different chapters. The map in Chapter 3 is adapted from Metzger et al. 2013.

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Chapter 2

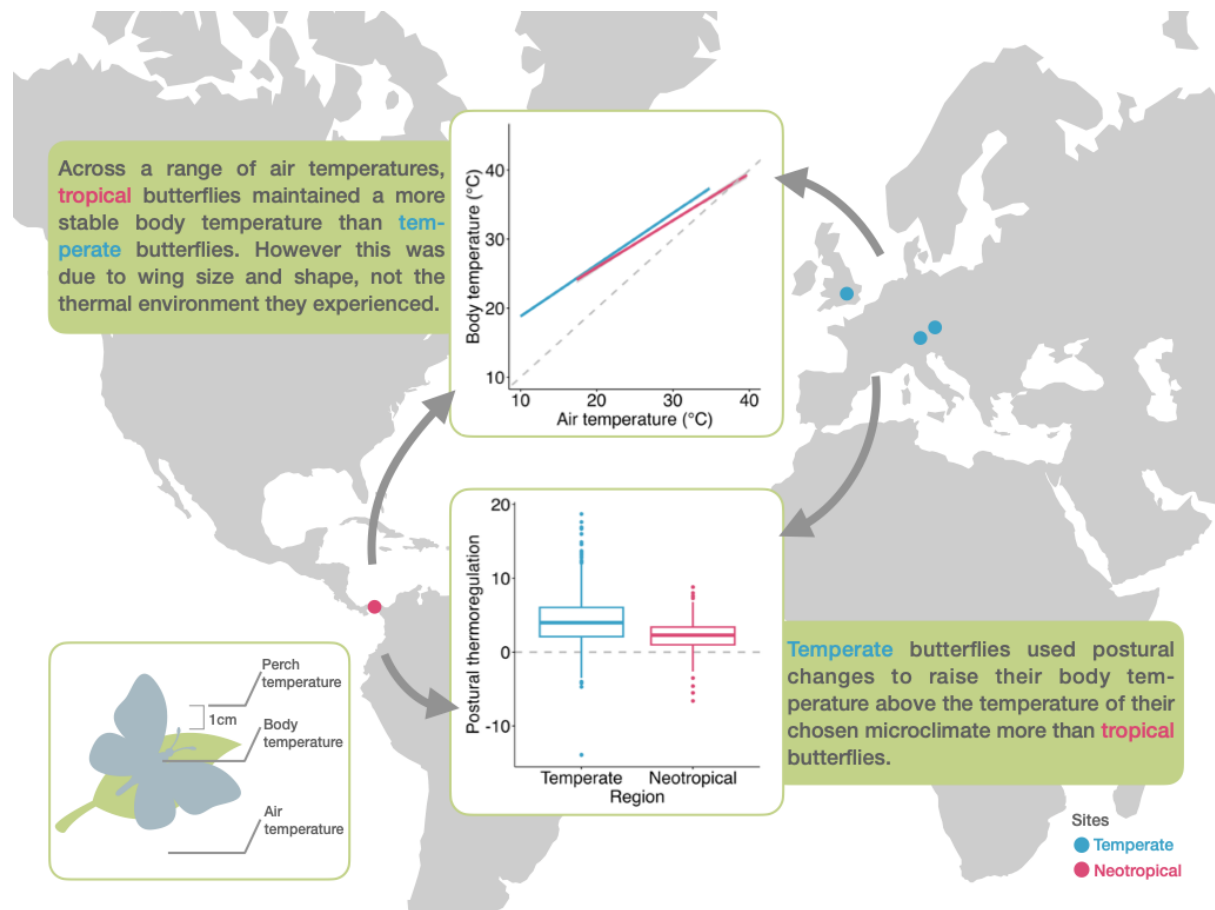
Thermoregulatory ability and mechanism do not differ consistently between neotropical and temperate butterflies

Laird-Hopkins, B. C., Ashe-Jepson, E., Basset, Y., Arizala Cobo, S., Eberhardt, L., Freiberga, I., Hellon, J., Hitchcock, G. E., Kleckova, I., Linke, D., Lamarre, G. P. A., McFarlane, A., Savage, A. F., Turner, E. C., Zamora, A. C., Sam, K., & Bladon, A. J. (2023). Thermoregulatory ability and mechanism do not differ consistently pattern neotropical and temperate butterflies. *Global Change Biology*, 00, 1-13. <https://doi.org/10.1111/gcb.16797>

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2.1 Abstract

Climate change is a major threat to species worldwide, yet it remains uncertain whether tropical or temperate species are more vulnerable to changing temperatures. To further our understanding of this, we used a standardised field protocol to (1) study the buffering ability (ability to regulate body temperature relative to surrounding air temperature) of neotropical (Panama) and temperate (the United Kingdom, Czech Republic and Austria) butterflies at the assemblage and family level, (2) determine if any differences in buffering ability were driven by morphological characteristics and (3) used ecologically relevant temperature measurements to investigate how butterflies use microclimates and behaviour to thermoregulate. We hypothesised that temperate butterflies would be better at buffering than neotropical butterflies as temperate species naturally experience a wider range of temperatures than their tropical counterparts. Contrary to our hypothesis, at the assemblage level, neotropical species (especially Nymphalidae) were better at buffering than temperate species, driven primarily by neotropical individuals cooling themselves more at higher air temperatures. Morphology was the main driver of differences in buffering ability between neotropical and temperate species as opposed to the thermal environment butterflies experienced. Temperate butterflies used postural thermoregulation to raise their body temperature more than neotropical butterflies, probably as an adaptation to temperate climates, but the selection of microclimates did not differ between regions. Our findings demonstrate that butterfly species have unique thermoregulatory strategies driven by behaviour and morphology, and that neotropical species are not likely to be more inherently vulnerable to warming than temperate species.



GRAPHICAL ABSTRACT 2.1

2.2 Introduction

Climate change is predicted to cause a rise in mean global temperatures, with larger increases at higher latitudes, as well as increases in the frequency of extreme temperature events (Lee et al., 2021). Temperature changes can cause shifts in physiology, morphology, life history, and distribution of species (Angilletta, 2009; Franco et al., 2006; Parmesan, 2006; Sunday et al., 2014). Considering this, the effect of temperature change on species is and will be wide-reaching, with potential cascading effects to communities and ecosystems (Parmesan, 2006; Sunday et al., 2014; Eggleton, 2020). Consequently, there is an urgent need to understand how species respond to changing temperatures.

To understand and predict the impacts of climate change on communities, it is important to identify the traits that affect species' sensitivity to temperature (Diamond et al., 2011; Diamond and Yilmaz, 2018). These include morphological traits, such as size or colouration, as well as physiological and behavioural traits, and may be a consequence of the thermal environment a species inhabits (Bonebrake et al., 2014; González-Tokman et al., 2020; Stella et al., 2018; Wenda et al., 2021). On a global scale, a major difference between thermal environments is found between temperate and tropical regions, with a narrower temperature range and higher mean annual temperature in tropical regions than temperate regions (Lee et al., 2021). Janzen (1967) predicted that the lower temperature variation found in tropical compared to temperate regions may select for narrower thermal tolerances among tropical species (the 'seasonality' hypothesis). Thus, although the degree of warming is predicted to be greater at higher latitudes, tropical species may be more at risk to temperature changes than temperate species, as they are less able to cope with variability (Lee et al., 2021). In addition, tropical species live closer to their thermal maximum than temperate species, and therefore may be more vulnerable to the same level of warming (Deutsch et al., 2008; Huey et al., 2009; Sunday et al., 2019). As a result, small variations in temperature would have severe consequences for the survival and fitness of tropical species (Deutsch et al., 2008; Ghalambor, 2006). However, other studies have shown that some temperate species may be equally vulnerable to climate change (Colado et al., 2022; Johansson et al., 2020; Mi et al., 2022). For example, temperate species with short activity periods may only experience narrow ranges in temperature (Johansson et al., 2020) and may be selected for low thermal tolerance

in a similar way to tropical species (Colado et al., 2022). Despite more than 50% of insects species being found in the tropics, there is a lack of systematic field studies investigating the thermoregulatory capabilities of tropical ectotherms, and comparing tropical and temperate species (Deutsch et al., 2008; Johansson et al., 2020; Stork, 2018; Sunday et al., 2012). It remains unclear the extent to which species from different ecological backgrounds and latitudes differ in their sensitivity to climate change.

The impact of climate change on species is often tested at the macroclimate scale, using coarse-scale weather station data, assuming that species live at these ambient temperatures (Diamond and Yilmaz, 2018; Pincebourde and Woods, 2020). This does not take into consideration the small-scale microclimate differences in temperature that individual species experience in heterogeneous environments, nor the fact that for small ectotherms moving just a small distance in these environments can result in exposure to very different climatic conditions (Pincebourde and Woods, 2020). Indeed, temperatures between microclimates and weather stations can differ significantly, with weather stations overestimating annual mean temperature in tropical understory forest by up to 0.5 °C and in highlands by up to 2.0 °C (Montejo-Kovacevich et al., 2020). Small-scale temperature variation can play an important role in species' thermal adaption, and can both buffer or magnify the effects of increasing temperatures (Pincebourde and Woods, 2020). We lack knowledge of how rising temperatures at the macroclimate scale impact microclimate temperatures, and how species are affected by these fine scale differences in temperature.

Butterflies are important as an indicator taxa, pollinators, prey and herbivores, but as ectotherms may be particularly vulnerable to temperature change (Bonebrake et al., 2010; Diamond and Yilmaz, 2018; Ma et al., 2021; Menéndez et al., 2007). Understanding how they respond to temperature change is crucial for assessing the ecosystem-wide impacts of climate change (Harvey et al., 2020). Butterflies can regulate their body temperature in a variety of ways, including by selecting favourable thermal microclimates during different life stages or at different times of day (Cómbita et al., 2022; De Frenne et al., 2021; Kearney et al., 2009; Kleckova et al., 2014). They also use other behavioural mechanisms, such as the orientation of their wings relative to the sun, to increase or reduce thermal absorbance (Kemp and Krockenberger, 2002; Shanks et al., 2015). Morphological characteristics, including wing

colour, reflectance, size and hair length, can also be important in determining thermoregulatory ability (Bonebrake et al., 2014; Stella et al., 2018; Wenda et al., 2021). For example, light coloured butterflies can regulate their body temperature by using their wings to reflect solar radiation onto their thorax to heat up, or back into the environment to cool down (Shanks et al., 2015; Zeuss et al., 2014). Dark-coloured butterflies can heat up quicker than light-coloured butterflies, perhaps explaining why they are found more frequently in cooler climates, such as forest interiors or higher latitudes, where being able to heat up quickly increases fitness (Günter et al., 2019; Xing et al., 2016). Size can also determine thermoregulatory ability, with the thermoregulation of sympatric mountain butterflies differing between species in relation to their habitat use and body size (Kleckova et al., 2014). Large species can use the surface area of their wings to intercept sunlight and are generally better at thermoregulating than small species (Bladon et al., 2020; Wenda et al., 2021). Finally, thermoregulatory ability differs systematically between butterfly families, with temperate Pieridae species being particularly good at thermoregulating compared to other temperate families (Bladon et al., 2020).

In this study, we compared buffering ability - the ability to regulate body temperature independently of the surrounding air temperature - of butterfly populations from neotropical (Panama) and temperate regions (Czech Republic, Austria, and the United Kingdom). We hypothesised (H1) that butterflies from temperate regions, being naturally exposed to a greater range of temperatures, will be better able to buffer their body temperature against changes in air temperature than those from the neotropical region. We tested this at the assemblage- and family- level. Since there was significant overlap in butterfly species found at our European sites, and at our Panamanian sites, we treated them as single assemblages, resulting in a temperate group and a neotropical group of the most common and conspicuous butterflies in each region. If differences in buffering ability between neotropical and temperate regions were observed we hypothesised (H2) that this will be driven by the temperatures butterflies are exposed to in the different regions, not by differences in their morphological traits. Finally, we hypothesised (H3) that as an adaptation to temperate climates, temperate butterflies will actively select warmer microclimates than neotropical butterflies, and that they will heat up through postural means more than neotropical species.

2.3 Methods

2.3.1 Study sites

Neotropical data were collected in Panama from February to June 2020 and from October 2021 to March 2022 during both wet (May to December) and dry (January to April) seasons (Supplementary Fig. 2.1, Supplementary Table 2.1) (Leigh, 1999). Temperate data were collected in the Czech Republic and Austria between April and August 2021 and in the United Kingdom between April and September 2009 and May and September 2018 (Supplementary Fig. 2.1, Supplementary Table 2.1) (Bladon et al., 2020). Data collection took place between 7:30 and 17:30. Neotropical field sites included lowland scrub and managed urban green spaces, secondary semi-deciduous lowland tropical forest, mountain rainforest and management agroforestry (Supplementary Table 2.1). Temperate field sites included calcareous meadows, grassland meadows, alpine/montane grassland, encroaching scrub, secondary forest, and exposed ground (Supplementary Table 2.1).

2.3.2 Butterfly body temperature and morphological measurements

Butterflies were captured with butterfly nets when encountered (without chasing) and data were collected following the protocol used by Bladon *et al.* (2020), as follows. Once in the net, and within 10s, a temperature reading of the butterfly's thorax (body temperature, T_b) was taken using a thermocouple (0.5 mm diameter) and handheld indicator (Tecpel Thermometer 305B, TC Direct). Air temperature (T_a) was taken at waist height where the butterfly was caught, with the thermocouple shaded from the sun. If the butterfly was resting on a substrate before capture, the temperature of the air 1 cm above where it was sat was recorded with the thermocouple (microclimate temperature, T_m). The butterfly was identified to species or subspecies. In the case of butterflies from the tropical *Calephelis* genus it was not possible to identify individuals to species, so data from these butterflies were aggregated to genus level. Forewing length (in mm) from the tip of the wing to the point where it meets the thorax was measured using callipers (at the Panama and UK sites only).

We calculated mean forewing length from our field data for each of the Panama and UK species. With the exception of *Erebia* spp., for which there was field data (Laird-Hopkins unpublished data), mean forewing lengths of butterflies from the Czech Republic and Austria were taken from the literature (Lindsey, 2016).

Wing aspect ratio (the ratio of forewing length : forewing width) strongly influences flight performance, which in turn can determine a species' capacity to move to more favourable microclimates and hence their ability to buffer air temperature (Chazot et al., 2016). Wing aspect ratio of all neotropical and temperate species was calculated from photographs sourced from the literature (Supplementary Table 2.2). To do this, photographs of five female and five male mounted specimens of each species were sourced and uploaded into ImageJ (Schneider et al., 2012). The forewing length (as above) and depth (longest line between the leading and trailing edges of the forewing, measured perpendicular to the wing length axis) were measured, and used to calculate forewing aspect ratio for each specimen. Mean aspect ratio was then calculated for each species. For sexually dimorphic species, the mean forewing aspect ratio of females and males was calculated separately.

Colour scale was determined for each species based on the lightness/darkness of their wings, following Bladon *et al.* (2020) (ranked: 1 white, 2 yellow-green, 3 orange, 4 orange-brown/blue, 5 brown, 6 black). If the difference in colour between males and females was sufficient to classify them in different categories, the mean colour category of females and males was used.

2.3.3 Statistical analysis

Preliminary analysis

To ensure our estimates of buffering ability were robust, only species with at least ten T_b measurements across a range of at least 5 °C of T_a were included in analyses. To estimate the buffering ability of each species, a simple linear regression with T_b as the response variable and T_a as the predictor variable was fitted separately for each species. The slope of the relationship was extracted and, following Bladon *et al.* (2020), subtracted from one to provide

an estimate of buffering ability (buffering ability estimate). With this method, a steep slope shows that the butterfly's T_b varies greatly over small ranges of T_a , thus indicating a low buffering ability. A shallow slope shows that the butterfly's T_b remains relatively stable over wide ranges of T_a , thus indicating a high buffering ability.

Do temperate and neotropical butterfly assemblages and families differ in buffering ability?

In total 94 species, across six families (Hesperiidae, Lycaenidae, Nymphalidae, Papilionidae, Pieridae and Riodinidae) were sampled in our study regions (neotropical: 54 species, $n = 1,333$; temperate: 40 species, $n = 5,482$). There is only one Riodinidae species (*Hamearis lucina*) found in Europe, so our analysis for this family specifically compared this species' buffering ability to those of its neotropical relatives. Papilionidae species were only sampled in the neotropical region, so this family was removed when comparing the buffering ability within families across neotropical and temperate regions.

To test for differences in buffering ability between butterfly assemblages, and within each butterfly family, in neotropical and temperate regions, linear mixed effects models were fitted for the assemblage and for each family separately. Body temperature was fitted as the response variable and T_a , region (temperate or neotropical) and their two-way interaction as predictor variables. Species was included as a random effect. A likelihood ratio test was performed to determine if the model with the two-way interaction was a better fit than the model without the term; if so, it would suggest that butterfly assemblages, or families, in the two regions differ in their buffering ability. The slope estimates for each region (neotropical and temperate) were extracted and subtracted from one as described above, to obtain a mean buffering ability estimate across each assemblage and family in each region.

When controlling for morphology, do temperate and neotropical butterflies differ in buffering ability?

Morphological characteristics are known to be important in determining species' thermoregulatory abilities (Bladon et al., 2020; Shanks et al., 2015; Sheldon and Tewksbury, 2014; Xing et al., 2016). We first determined if morphological characteristics (mean forewing length, mean forewing aspect ratio and wing colour) differed between neotropical and

temperate species. We fitted one-way ANOVAs with mean forewing length (mm), mean wing aspect ratio and wing colour separately as the response variable and region (neotropical or temperate) as the predictor variable.

To determine if forewing length, forewing aspect ratio and/or wing colour were driving differences in buffering ability between neotropical and temperate butterflies we fitted linear mixed effects models. Body temperature (T_b) was the response variable. Air temperature (T_a), region (temperate or neotropical), mean forewing length, mean forewing aspect ratio and wing colour, and the two-way interactions between T_a and each of the other terms were predictor variables. Mean forewing length was $\log_{10}$ -transformed to ensure all predictor variables were on a similar scale. Species was included as a random effect. If the interaction between T_a and region was retained in the best fitting model, it would suggest that buffering ability differs between regional assemblages in addition to any effect of morphology. To find the best fitting model we used backwards stepwise selection with the step function in R package *lmerTest* (Kuznetsova et al., 2017). A preliminary Pearson correlation coefficient analysis indicated no correlation between the three continuous predictor variables (mean forewing length, mean forewing aspect ratio and wing colour), justifying their inclusion as predictor variables in the same models (Supplementary Panel 2.1). Preliminary analysis fitting one-way ANOVAs with mean forewing length (mm), mean wing aspect ratio and wing colour separately as the response variable and family as the predictor variable showed that there was a significant effect of family on the three response variables (Supplementary Table 2.3). Family was therefore not included in the linear mixed effects model described above.

Do temperate and neotropical butterfly assemblage and families differ in their use of microclimate selection and postural thermoregulation, or in their index of thermal specialisation?

The data were subset so that only species with at least ten microclimate temperature measurements (T_m) were included in this analysis. For the assemblage-level analysis, this included 39 species, 13 neotropical ($n = 176$; families: five Hesperiidae, seven Nymphalidae and one Riodinidae) and 26 temperate ($n = 919$; families: three Hesperiidae, four Lycaenidae, 15 Nymphalidae and four Pieridae) (Supplementary Table 2.4). For the family-level analysis

there were only sufficient data from both temperate and neotropical regions for Hesperiidae and Nymphalidae to undertake this analysis. This included five neotropical and three temperate Hesperiidae (neotropical: $n = 57$; temperate: $n = 56$), and seven neotropical and 15 temperate Nymphalidae (neotropical: $n = 101$; temperate: $n = 499$).

For each individual butterfly, we calculated the extent to which its chosen T_m differed from the surrounding T_a , by subtracting T_a from T_m (“microclimate selection”, following Bladon *et al.* (2020)). A large difference between T_m and T_a indicates that the butterfly is selecting a specific thermal microclimate distinct from T_a . For each butterfly, we also calculated the extent to which T_b differed from the temperature of its chosen T_m , by subtracting T_m from T_b (“postural thermoregulation”, following Bladon *et al.* (2020)). A large difference between T_m and T_b suggests that the butterfly is regulating its temperature independently of its chosen microclimate, for example by behavioural posturing relative to the sun. To quantify the relative contribution of microclimate selection and postural thermoregulation to each butterfly’s body temperature, we calculated an index of thermal specialisation (ITS) by subtracting microclimate selection ($T_m - T_a$) from postural thermoregulation ($T_b - T_m$). A large ITS value indicates that the butterfly is utilising postural thermoregulation more, and is able to alter its body temperature beyond that of its immediate environment, making it a thermal generalist. A small ITS value means that the butterfly relies more on microclimate selection for thermoregulation, and is a thermal specialist (Bladon *et al.* 2020).

To test whether the use of microclimate selection and postural thermoregulation, and the value of ITS, differed between the butterfly assemblage and within butterfly families from neotropical and temperate regions, linear mixed effects models were fitted for the assemblage, and each family separately. Microclimate selection, postural thermoregulation and ITS were fitted separately as response variables, and region (temperate or neotropical), T_a and their two-way interaction were included as predictor variables. Species was included as a random effect. A likelihood ratio test was performed to determine if the full model was a better fit for the data than a model without the interaction term between region and T_a ; if so, it would suggest that butterflies from different regions differed in their use of microclimate selection, postural thermoregulation or ITS across a range of T_a . An additional likelihood ratio test was performed to determine if the model including region and T_a as a predictor variable

was a better fit than the model including only T_a ; if so, it would suggest that butterflies from different regions differed in their use of microclimate selection, postural thermoregulation or ITS, but independently of T_a .

To control for differences in T_a between neotropical and temperate regions and to assess the robustness of our results, all analyses were repeated on a subset of data that were restricted to the same T_a range in both neotropical and temperate regions. All statistical analyses were undertaken in R version 4.1.2 (R Core Team, 2019). Graphics were produced using the package *ggplot2* (Wickham, 2016). Linear mixed effects models were fitted using the *lme4* package (Bates et al., 2015). To ensure normality and homogeneity in the residuals, graphical model diagnostics were applied to each model. The response parameter was $\log_{10}$ -transformed where necessary to achieve normality. Marginal means for neotropical and temperate species and families were predicted using the package *ggeffect* (Lüdtke, 2018). The marginal effects of region, i.e., the difference between marginal means, were used as a measure of effect size at the response scale.

2.4 Results

From the neotropical region, 1,333 individuals from 54 species were measured. Air temperature (T_a) ranged from 17.4 °C to 39.7 °C, with a mean of 28.5 °C. Body temperature (T_b) ranged from 22.1 °C to 40.3 °C, with a mean of 31.7 °C. From the temperate region, 5,482 individuals of 40 species were measured (Supplementary Results 2.1). T_a ranged from 10.0 °C to 34.8 °C, with a mean of 22.2 °C. T_b ranged from 15.4 °C to 45.0 °C, with a mean of 28.0 °C. The buffering ability estimates for neotropical species ranged from -0.317 (*Hemiargus hanna*) to 1.005 (*Phoebis argante*), with a mean of 0.295 (Supplementary Fig. 2.2, Supplementary Table 2.4 and 2.5). The buffering ability estimates for temperate species ranged from -0.404 (*Hamearis lucina*) to 0.675 (*Erebia medusa*), with a mean of 0.243 (Supplementary Fig. 2.2, Supplementary Table 2.4).

2.4.1 Do temperate and neotropical butterfly assemblages differ in buffering ability?

Across the whole assemblage, the mean buffering ability of neotropical butterflies (0.350 ± 0.035) was significantly higher than the buffering ability of temperate butterflies (0.220 ± 0.010), with neotropical species appearing better able to reduce their T_b at higher T_a ($\chi^2 = 14.17$, $df = 1$, $p < 0.001$; Fig. 2.1). This means that across a 20.0°C range of T_a , predicted neotropical butterfly T_b would vary by 13.0°C , while predicted temperate butterfly T_b would vary by 15.6°C . A similar result was obtained when data were restricted to the range of T_a which occurred in both regions (Supplementary Fig. 2.3 and Supplementary Results 2.2).

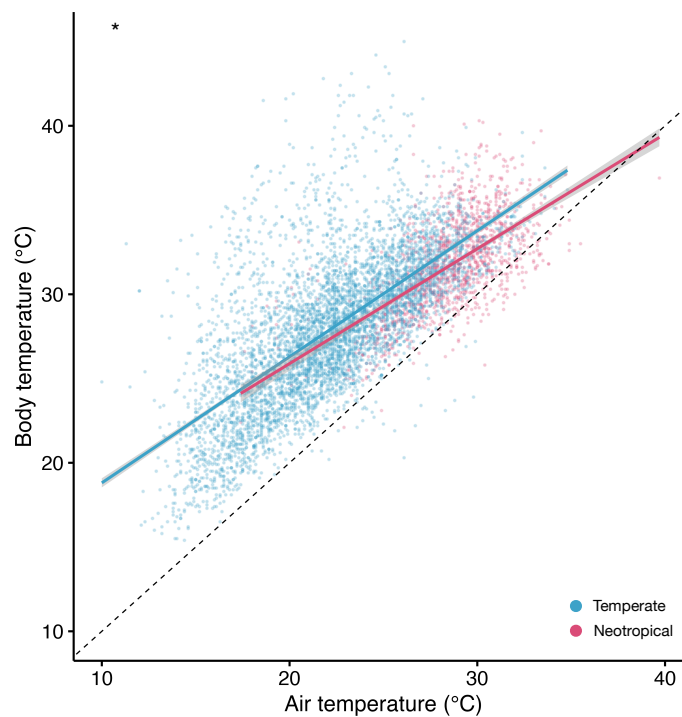


FIGURE 2.1. Individual butterfly body temperatures ($^{\circ}\text{C}$) at different air temperatures ($^{\circ}\text{C}$) from neotropical (pink) and temperate (blue) regions. Solid lines represent the modelled relationship between body temperature and air temperature. The grey bands show 95% confidence intervals. The black dashed line represents a 1:1 relationship between body and air temperature. The asterisk marks the significant difference in buffering ability estimate between neotropical and temperate butterflies.

2.4.2 Do temperate and neotropical butterfly families differ in buffering ability?

In the neotropical region, the buffering ability of butterfly families ranged from -0.020 (Lycaenidae) to 0.426 (Pieridae). This means that across a 20.0°C range of T_a , predicted neotropical Lycaenidae T_b would vary by 20.4°C , while predicted neotropical Pieridae T_b would vary by 17.2°C . In the temperate region, family-level buffering ability ranged from -

0.404 (Riodinidae) to 0.326 (Pieridae) (Supplementary Table 2.6). This means that across a 20.0 °C range of T_a , predicted temperate Riodinidae T_b would vary by 28.1 °C, while predicted temperate Pieridae T_b would vary by 20.2 °C. Neotropical Nymphalidae were better at buffering their T_b against changes in T_a than Nymphalidae from temperate regions ($\chi^2 = 21.66$, $df = 1$, $p < 0.001$), and Riodinidae from neotropical regions were better at buffering their T_b against changes in T_a than *Hamearis lucina*, the only temperate Riodinidae species ($\chi^2 = 11.83$, $df = 1$, $p < 0.001$). This was driven by neotropical Nymphalidae and Riodinidae being better able to lower their T_b at high T_a compared to temperate Nymphalidae and Riodinidae (Fig. 2.2). This means that across a 20 °C range of T_a , predicted temperate and neotropical Nymphalidae T_b would vary by 16.4 °C and 12.1 °C, respectively, and predicted temperate and neotropical Riodinidae T_b would vary by 28.1 °C and 12.5 °C, respectively. There were no differences in family-level buffering ability between temperate and neotropical species for the other three families (Fig. 2.2, Supplementary Table 2.6).

When data were restricted to the range of air temperatures which occurred in both regions, a similar result was obtained for Nymphalidae and Pieridae, while Hesperidae and Lycaenidae showed better buffering ability in temperate than neotropical regions (Supplementary Fig. 2.4 and Supplementary Table 2.7). Specifically, temperate Hesperidae and Lycaenidae increased their T_b at low T_a more so than neotropical Hesperidae and Lycaenidae. It was not possible to model the restricted dataset for Riodinidae, due to limited temperature overlap between regions for the dataset (Supplementary Results 2.3).

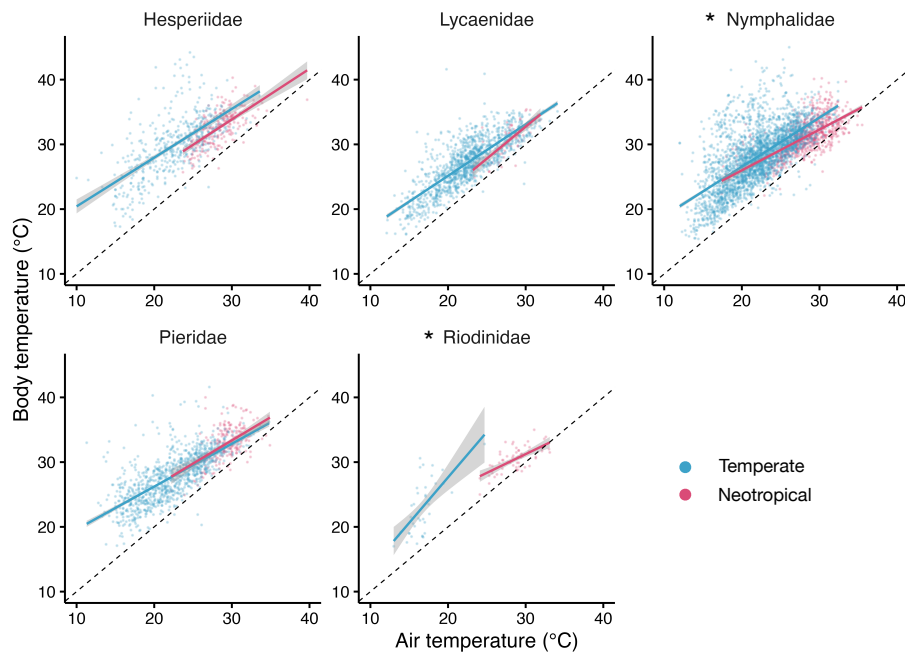


FIGURE 2.2. Individual butterfly body temperatures (°C) at different air temperatures (°C) from neotropical (pink) and temperate (blue) regions for each taxonomic family. Solid lines represent the modelled relationship between body temperature and air temperature. The grey bands show the 95% confidence intervals. The black dashed line represents a 1:1 relationship between body and air temperature. Families with a significant difference in buffering ability estimate between neotropical and temperate butterflies are marked with an asterisk.

2.4.3 When controlling for morphology do temperate and neotropical butterflies differ in buffering ability?

Mean forewing length was significantly larger in neotropical butterflies (26.7 ± 0.3 mm) than in temperate butterflies (19.9 ± 0.1 mm) ($F = 581.75$, $df = 1$, $p < 0.001$). Neotropical butterflies had a significantly higher mean wing aspect ratio (1.78 ± 0.01) than butterflies from temperate regions (1.71 ± 0.00) ($F = 233.85$, $df = 1$, $p < 0.001$). Butterflies from neotropical regions were significantly darker (mean colour scale = 4.12 ± 0.04) than temperate butterflies (mean colour scale = 3.55 ± 0.02) ($F = 229.43$, $df = 1$, $p < 0.001$). The best fitting model included T_a , region (temperate or neotropical), mean forewing length and mean forewing aspect ratio, and the two-way interactions between T_a and each of mean forewing length and mean forewing aspect ratio. This showed that there was no difference in buffering ability between neotropical and temperate butterflies when accounting for differences in morphology (Table 2.1). Differences in mean forewing length and mean forewing aspect ratio were the main drivers of differences in buffering ability between neotropical and temperate butterflies (Table 2.1). Butterflies with larger mean forewing lengths and higher mean wing aspect ratios (longer,

shallower wings) were better at buffering than their T_b against changes in T_a than those with smaller mean forewing lengths and lower mean wing aspect ratios (shorter, deeper wings) (Table 2.1). The same result was found when the data were restricted to the range of air temperatures which occurred in both regions (Supplementary Table 2.8).

TABLE 2.1. Output from the best fitting linear mixed effects model, with body temperature (T_b) as the predictor variable and air temperature (T_a), region (neotropical and temperate), mean forewing length, mean forewing aspect ratio and the two-way interaction between T_a and each of mean forewing length and mean forewing aspect ratio as response variables. Mean forewing length was log10 transformed to ensure it was on the same scale as other predictor variables. Species was included as a random effect. Significant terms are marked with an asterisk.

	Estimate	SE	df	F	<i>p</i>
Region: temperate	-20.196	4.590	1	22.137	<0.001*
Region: tropical	-21.662	0.312			
T_a	1.792	0.156	1	132.181	<0.001*
Mean forewing length	6.040	0.903	1	44.770	<0.001*
Mean forewing aspect ratio	7.728	2.247	1	11.827	<0.001*
T_a : Mean forewing length	-0.456	0.070	1	42.623	<0.001*
T_a : Mean forewing aspect ratio	-0.252	0.763	1	10.947	<0.001*

2.4.4 Do temperate and neotropical butterfly assemblages and families differ in their use of microclimate selection and postural thermoregulation, or in their index of thermal specialisation?

This analysis included a total of 26 temperate ($n = 919$) and 13 neotropical ($n = 176$) species. The use of microclimate selection was not significantly different between temperate and neotropical species ($\chi^2 = 0.104$, $df = 1$, $p = 0.747$) (Fig. 2.13a, Supplementary Table 2.9). This result was the same for the two families tested individually (Hesperiidae: $\chi^2 = 0.025$, $df = 1$, $p = 0.875$; Nymphalidae: $\chi^2 = 0.007$, $df = 1$, $p = 0.932$). The use of postural thermoregulation was greater in temperate species than in neotropical ($\chi^2 = 8.314$, $df = 1$, $p = 0.004$), however when testing families individually only Nymphalidae reflected this result ($\chi^2 = 11.86$, $df = 1$, $p < 0.001$); there was no difference between neotropical and temperate Hesperiidae ($\chi^2 = 2.431$, $df = 1$, $p = 0.119$) (Fig. 2.3b, Supplementary Table 2.9). Temperate species used postural thermoregulation to increase their body temperature by a mean of 4.2 ± 0.1 °C compared to 2.2 ± 0.2 °C in neotropical species. Temperate Nymphalidae used postural thermoregulation to increase their body temperature by a mean of 4.7 ± 0.2 °C compared to 2.1 ± 0.2 °C in neotropical Nymphalidae. The index of thermal specialisation (ITS) was greater in temperate

than neotropical species ($\chi^2 = 7.150$, $df = 1$, $p = 0.008$) and when testing families individually the same was found in Nymphalidae ($\chi^2 = 7.970$, $df = 1$, $p = 0.005$), but not HesperIIDae ($\chi^2 = 2.516$, $df = 1$, $p = 0.113$) (Fig. 2.3c, Supplementary Table 2.9). Temperate species ITS was 2.9 ± 0.1 °C compared to 1.4 ± 0.3 °C in neotropical species and temperate Nymphalidae ITS was 3.3 ± 0.2 °C compared to 1.2 ± 0.3 °C in neotropical Nymphalidae. There was no effect of air temperature on the difference in how butterflies from neotropical and temperate regions used microclimate selection, postural thermoregulation or ITS, across the whole assemblage and between the two families tested individually (HesperIIDae and Nymphalidae) (Supplementary Table 2.9).

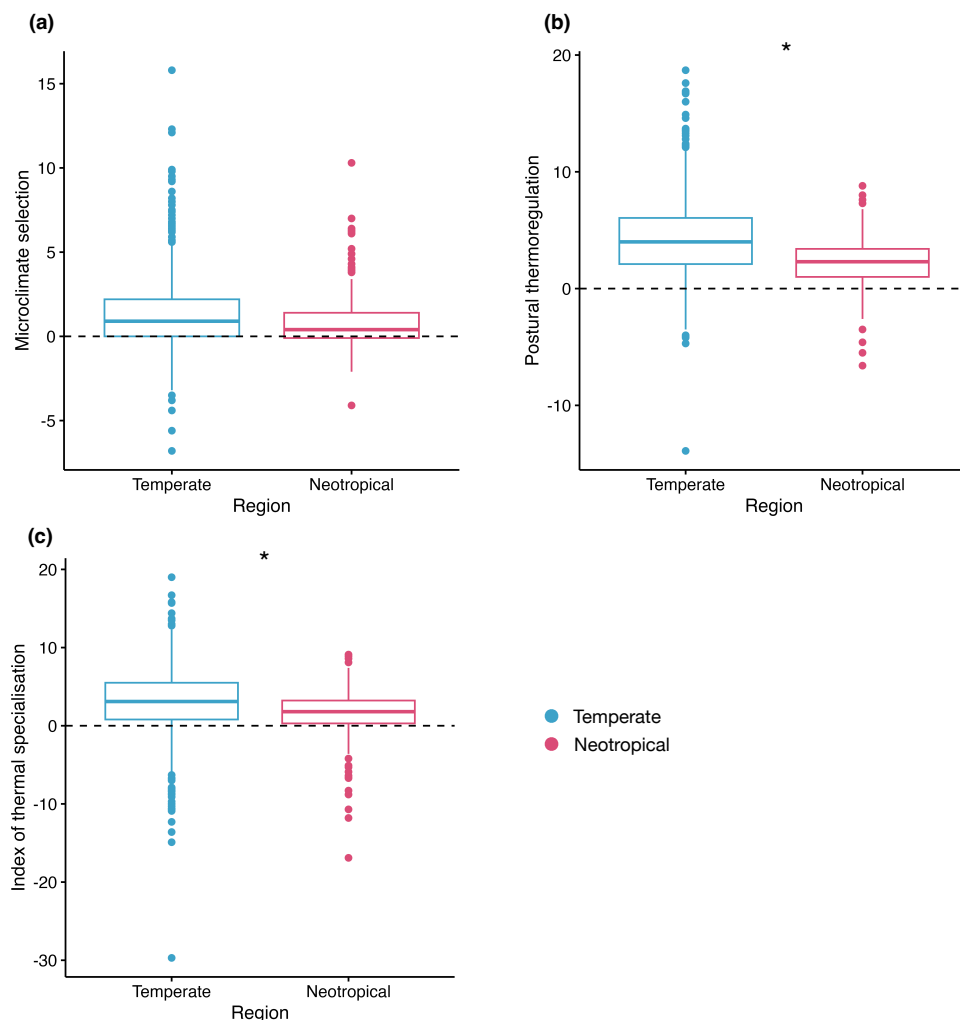


FIGURE 2.3. *Microclimate selection (the difference between microclimate temperature and air temperature, (a), postural thermoregulation (the difference between body temperature and microclimate temperature, (b) and index of thermal specialisation (postural thermoregulation – microclimate selection, (c) of temperate (blue) and neotropical (pink) butterflies. Significant differences between neotropical and temperate butterflies are marked with an asterisk.*

When data were restricted to the range of air temperatures which occurred in both regions, there was no difference in the use of microclimate selection, postural thermoregulation or ITS between butterflies from neotropical and temperate regions (Supplementary Results 2.4, Supplementary Fig. 2.5 and Supplementary Table 2.10). Microclimate selection and ITS did not differ significantly between neotropical and temperate Nymphalidae, but temperate Nymphalidae used postural thermoregulation more than neotropical Nymphalidae (Supplementary Results 2.4, Supplementary Fig. 2.5 and Supplementary Table 2.10). There was insufficient data to undertake this analysis for the other families.

2.5 Discussion

We found that at the assemblage level, neotropical butterflies are better at buffering their body temperature against changes in air temperature than temperate butterflies. At the family level the same trend was found in Nymphalidae and in the comparison of the three neotropical Riodinidae species to the single European species, but there was no difference for Lycaenidae, Pieridae or Hesperiiidae. In the dataset restricted to the range of air temperatures overlapping in both regions, temperate Hesperiiidae and Lycaenidae showed better buffering ability than their neotropical counterparts. When controlling for morphological characteristics, there was no difference in buffering ability between the two regions, but larger species and those with higher wing aspect ratios (longer, shallower wings) were better at buffering their body temperature against changes in air temperature than smaller species and those with lower wing aspect ratios (shorter, deeper wings). There were no differences in the use of microclimate selection for thermoregulation between butterflies from neotropical and temperate regions at the assemblage or family level. At the assemblage level, postural thermoregulation was used more to control temperature by temperate than neotropical species, and this was also true for Nymphalidae, but not for Hesperiiidae, at the family level. Overall, the index of thermal specialisation was greater in temperate butterflies than in neotropical butterflies, but at the family level this was only the case for Nymphalidae and not Hesperiiidae.

2.5.1 Thermal buffering ability

Across the whole assemblage, neotropical butterflies had a better buffering ability than temperate butterflies, with neotropical species having relatively cooler body temperatures at higher air temperatures than temperate species. At the family level, only Nymphalidae and Riodinidae showed a difference in buffering ability between neotropical and temperate regions, with neotropical Nymphalidae and Riodinidae species being better at buffering compared to their temperate counterparts. However, as there was only one temperate and three neotropical Riodinidae species in our dataset, it is not possible to make general assumptions about the thermoregulatory capacities of butterflies from this family, and our finding likely reflects the poor buffering ability of *Hamearis lucina*, the only European species. As half of the species sampled in both temperate and neotropical regions were from the Nymphalidae family (47 out of 91 species) it is likely that this family was driving the trend observed at the assemblage level. Notably, this result was the same when data were restricted to the range of air temperatures which occurred in both regions, indicating that this is a pattern which is independent of the specific range of temperatures butterflies experienced during this study. However, in the restricted dataset, temperate HesperIIDae and Lycaenidae species showed better buffering ability than their neotropical counterparts, suggesting that they were better able to cope with changes in air temperature. This result was likely driven by temperate butterflies warming up more at a given air temperature, in an effort to increase activity in the cooler, temperate environment.

The buffering ability response from the dataset including the full range of air temperatures was driven by neotropical species cooling down more than temperate species at high air temperatures. Indeed, at the species level, some neotropical species maintained their body temperature at temperatures lower than air temperature, when temperatures were high (e.g. *Heliconius hecale melicerta*). There is likely to be strong selection among neotropical species to avoid high temperatures, as tropical species live closer to their thermal safety margin (Deutsch et al., 2008; Sunday et al., 2019, 2014, 2012). The better buffering ability of neotropical butterflies may be further explained by differences in habitat type between the neotropical and temperate regions. There was more tree cover and shade in the neotropical sites compared to the temperate sites, and hence a greater availability of cool microclimates.

At high air temperatures, neotropical butterflies may use these marginally cooler microclimates to lower their body temperature, avoiding their thermal limit and the associated decrease in survival and fitness.

It is important to note that our analysis only included data for 54 of 601 neotropical species found in the region (Basset et al., 2015), and 40 of 496 temperate species (Wiemers et al., 2018). This means our results may not be representative of the entire tropical and temperate butterfly assemblages found in the wider regions. However, the neotropical species we sampled are not endemic to Panama and are found elsewhere in the neotropics, making our tropical data likely to be a reliable subsample of the wider neotropical butterfly community. It is also possible that the species we surveyed have a higher than average thermoregulatory ability for their assemblage, as butterflies which thermoregulate efficiently are able to be more active, hence are more likely to be seen and sampled. Nonetheless, we sampled a large pool of species at random, which is likely to reduce the chance of this bias affecting results, and any bias for abundant or conspicuous species is likely to be similar in both regions. Finally, the differences in buffering ability we recorded between regions could be the result of evolutionary history and genetic drift, rather than selection pressures, as neotropical and temperate butterflies diverged a long time ago. However, by undertaking family-level analysis we partly control for phylogeny driven differences in buffering ability. Additionally, thermoregulation is a highly important factor in butterfly ecology, so traits related to temperature control are likely to be under strong selection pressure.

2.5.2 The importance of morphological traits

Alongside the better buffering ability of neotropical species at the assemblage level, we found that neotropical butterflies are generally larger and darker and have a higher wing aspect ratio (longer, shallower wings) than temperate butterflies. Larger species and those with higher wing aspect ratios had a better buffering ability than smaller species and those with lower wing aspect ratios (shorter, deeper wings). This reflects similar results elsewhere. For example, large temperate butterflies buffer air temperature changes better than small butterflies (Bladon et al., 2020), because their larger wing surface area allows them to better regulate their temperature by basking (Kingsolver, 1988; Shanks et al., 2015; Wasserthal,

1975; Xing et al., 2016; Zeuss et al., 2014). Additionally, large butterflies may be more mobile and able to search out cooler microclimates, allowing them to buffer against high air temperatures (Chazot et al., 2016). High wing aspect ratio is associated with greater mobility and flying speed (Hassall, 2015). As far as we are aware it is unknown how wing aspect ratio impacts thermoregulatory ability, however greater mobility may enable neotropical butterflies to search out favourable microclimates more effectively. Therefore, the better buffering ability of neotropical butterflies compared to temperate butterflies in our dataset could be due to either the larger wing length or higher wing aspect ratio of the neotropical species. However, when these effects were controlled for, the difference in buffering ability between regions did not persist.

2.5.3 Microclimate selection, postural thermoregulation, and index of thermal specialisation

We found that there was no difference in the use of thermoregulation via microclimate selection between neotropical and temperate species, but temperate species used postural thermoregulation to raise their body temperature more than neotropical species. Neotropical species generally maintained their body temperature closer to ambient air temperature than temperate species. Using postural thermoregulation to heat up is likely to be an adaptation to cooler climates. Butterflies require heat to become active, and lose heat as they fly (Advani et al., 2019). When there is a large difference between air temperature and optimal body temperature, as in temperate systems, heat loss during flight is greater, and so temperate species may be under stronger selection than neotropical species to use postural thermoregulation to heat up, enabling them to become active and increase flight duration in cooler air temperatures. Indeed, temperate butterflies from higher latitudes take-off at higher body temperatures than butterflies from lower latitudes (Advani et al., 2019). For temperate species in our study, undertaking postural thermoregulation to gain more heat before take-off might compensate for the additional heat lost during flight at lower air temperatures, which could increase activity and survival. This result could also be due to other morphological differences between neotropical and temperate butterflies, such as colour, with lighter species, which were found more commonly in the temperate than neotropical study sites, being able to undertake postural thermoregulation better than darker species. Alternatively,

it may be a consequence of the different habitat types that were sampled between neotropical and temperate regions. In the temperate region more open habitats were sampled, which would have provided greater opportunities for basking and could explain why temperate butterflies undertook postural thermoregulation more than neotropical butterflies.

We found that neotropical species were more thermally specialist, relying relatively more on the selection of available microclimates than postural thermoregulation, when compared to temperate species. However, this result is driven by temperate species undertaking postural thermoregulation to increase their body temperature. Neotropical species maintained their body temperature closer to ambient temperature than temperate species did, especially at higher air temperatures, presumably as a mechanism to avoid overheating. This could make them vulnerable to extreme heatwave events, as, if air temperature increases, their ability to avoid temperatures outside their thermal safety margin might become limited.

At the family level, we found the same result in Nymphalidae, the most abundant family in our dataset, but not Hesperidae, so Nymphalidae were likely driving the assemblage-level trend. Nymphalidae species are often larger than those in other butterfly families, and it is possible that their wing size enables them to undertake postural thermoregulation more effectively, as larger wings can better control temperature through basking than smaller wings.

2.5.4 Implications

Climate change, through increasing temperatures, is expected to negatively affect ectotherms, decreasing their performance and, in extreme cases, resulting in extinction (Diamond and Yilmaz, 2018; Johansson et al., 2020; Mi et al., 2022). By buffering their body temperature against changes in air temperature using a combination of microclimate selection and postural thermoregulation, butterflies might be able to mitigate some of these negative effects. We found that neotropical butterfly species, which may be living closer to their thermal limit than temperate species, buffer their body temperature against changes in air temperature better than temperate species, primarily by cooling down more at higher air temperatures (Deutsch et al., 2008; Sunday et al., 2019, 2014, 2012). In the restricted dataset, temperate Hesperidae

and Lycaenidae were better at buffering their body temperature against changes in air temperature than their neotropical counterparts, but this was driven by their ability to warm up more at a given air temperature. Similarly, temperate butterflies, and in particular Nymphalidae, were better at using behaviour for thermoregulation than neotropical butterflies, but this is likely to be an adaptation for warming up in cool temperate climates, and may not indicate a good ability to cool down under rising temperatures. Therefore, both neotropical and temperate butterfly species show signs of vulnerability to rising temperatures, with no overriding ecological difference in sensitivity to climate change between bioclimatic regions. However, the degree to which physiological acclimation or genetic adaptation could offset the negative effects of increasing temperatures on these butterflies remains unclear (Diamond and Yilmaz, 2018; Seebacher et al., 2015; Sgrò et al., 2016; Sheldon and Tewksbury, 2014).

Our study has focused solely on the adult stage, but thermal sensitivity and hence vulnerability to changing temperatures varies markedly between life stages (Kingsolver et al., 2011). Indeed, life stage is the most important factor for predicting tolerance to changes in temperature, and mortality mostly occurs before the adult stage (Bowler and Terblanche, 2008). Eggs are thought to be the most vulnerable life stage, compared to larvae, pupae and adults, as a consequence of their physiology and their immobility, which means they cannot locate more favourable microclimates (Kingsolver et al., 2011). Larvae are also likely to be sensitive to increasing temperatures, as although mobile, their dispersal ability is limited (Kingsolver et al., 2011). Additionally, most Lepidoptera spend more time in the larval than the adult stage, meaning temperature changes are more likely to impact this life stage. Therefore, future studies should investigate the buffering ability and thermoregulatory mechanisms of all butterfly life stages.

Although microclimates may offer thermal refugia for adult Lepidoptera, the extent to which they will benefit less mobile life stages with regards to temperature control and coping with temperature extremes remains unclear (Montejo-Kovacevich et al., 2020). Habitat degradation and land-use change has been shown to decrease the availability of cooler microclimates and hence the temperature buffering potential of a habitat (Jucker et al., 2020). As conversion of land for agricultural use continues to accelerate and global temperatures

rise, the protection of pristine habitats for their temperature buffering potential may become increasingly important.

2.5.5 Conclusion

To our knowledge, this is the first study comparing the thermal buffering ability of ectotherms between tropical and temperate systems. By using standardised methods to measure buffering ability and mechanisms, we provide a template for making cross-latitudinal comparisons within taxa, increasing understanding of how species across latitudes cope with changing temperatures. We stress the need for further cross-latitudinal studies comparing the thermal capabilities of tropical and temperate species. Despite over half of the world's insect species living in tropical systems, most research focuses on the thermal capabilities of temperate ectotherms (Garcia-Robledo et al., 2020; Stork, 2018). Future studies should focus on tropical ectotherms and expand knowledge of how these species regulate temperature and their potential vulnerabilities to climate change. We also highlight the importance of considering species' morphological traits, and use of microclimate selection and postural thermoregulation when predicting the vulnerability of tropical and temperate species to future climate change. Future studies should also investigate the role of habitat structure in the buffering ability of butterflies, for example determining how favourable microclimates may act as refuges from extreme temperatures for tropical and temperate species, paving the way to informing management options that could protect species from warming. Most importantly, our results show that individual species have their own individual thermoregulatory capabilities. We stress the need for caution when making generalised assumptions of species' vulnerability to climate change based solely on the thermal environment they inhabit.

2.6 Bibliography

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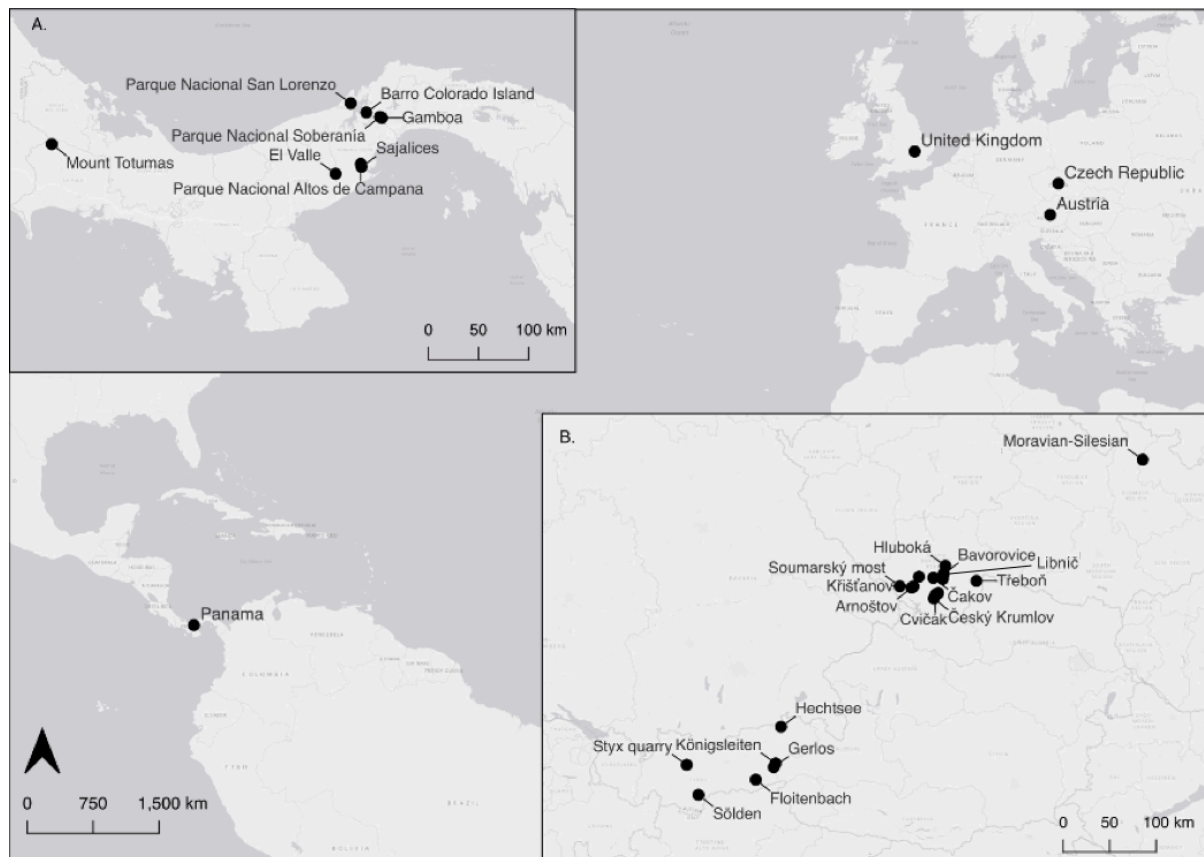
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2.7 Supplementary material



SUPPLEMENTARY FIGURE 2.1. Broad study locations in Panama, Austria, the Czech Republic and the UK (main figure) with insets of field sites in Panama (neotropical, inset A) and Austria and the Czech Republic (temperate, inset B) (Supplementary Table 2.1) (ESRI, 2022). See Bladon et al. 2020 for the location of United Kingdom field sites.

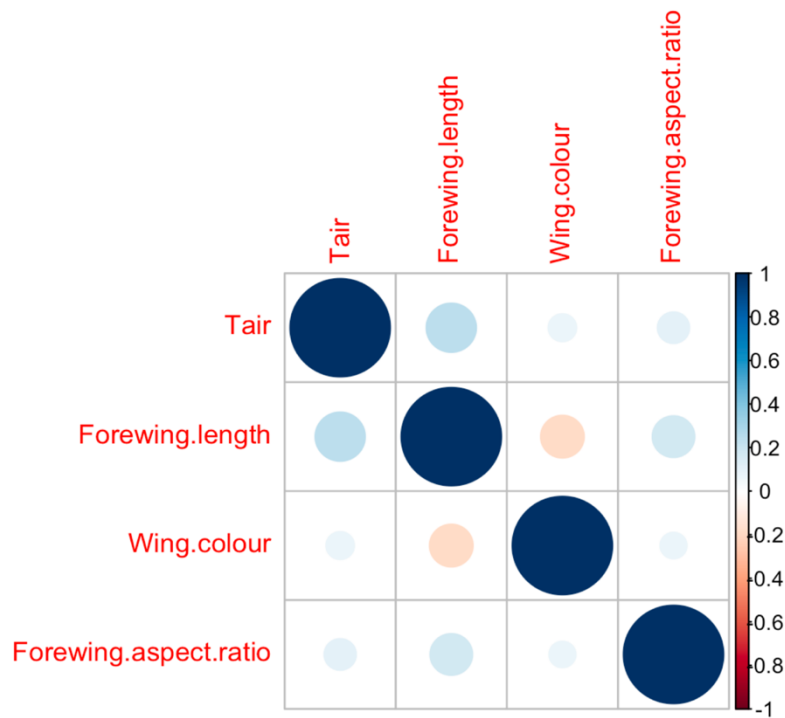
SUPPLEMENTARY TABLE 2.1. *Study sites in Austria, the Czech Republic, the United Kingdom and Panama, including coordinates, elevation (meters above sea level) and land type.*

Site	Coordinates		Elevation	Land type
	N	E		
TEMPERATE Austria				
Floitenbach	47.099153	11.810562	996	Exposed ground, alpine grassland meadows, encroaching scrub and secondary forest
Gerlos	47.218297	12.062329	1346	Exposed ground, alpine grassland meadows and forest edge
Hechtsee	47.599623	12.164717	670	Forest edge
Königsleiten	47.253974	12.089779	2050	High alpine grassland meadow
Sölden	46.954529	11.008711	1511	Exposed ground, alpine grassland meadows, encroaching scrub and secondary forest
Styx quarry	47.239532	10.844794	733	Exposed ground, alpine grassland meadows, forest edge and secondary forest
TEMPERATE Czech Republic				
Arnoštov	48.894660	13.994615	840	Grassland meadows
Bavorovice	49.023401	14.443310	375	Grassland meadows
Čakov	48.984240	14.296788	426	Grassland meadows
České Budějovice	48.974414	14.430564	442	Grassland meadows and secondary forest edge
Český Krumlov	48.793907	14.304518	503	Grassland meadows, roadside verges, secondary forest
Cvičák	48.829148	14.326293	591	Grassland meadows, encroaching scrub and marshland
Hluboká	49.094034	14.465941	405	Grassland meadows and secondary forest
Křišťanov	48.906300	14.019557	940	Grassland meadows
Libníč	49.014427	14.547689	333	Grassland meadows
Moravian-Silesian	50.056424	17.228311	1444	Grassland meadows
Rájov	48.843466	14.365909	459	Grassland meadows
Soumarský most	48.907138	13.827730	754	Grassland meadows and secondary forest
Třeboň	48.955848	14.900563	446	Grassland meadows
Zelený	48.993960	14.096682	805	Grassland meadows
TEMPERATE United Kingdom				
Ben Lawers	56.532870	-4.273260	614	Montane grassland
Blows Downs	51.883210	-0.495800	202	Calcareous grassland meadows and scrub
Haweswater	54.507560	-2.845980	652	Montane grassland
Irton Fell	54.406720	-3.340000	236	Montane grassland
Pegsdon Hills	51.953540	-0.370200	100	Calcareous grassland meadows and scrub
Totternhoe Knolls	51.889890	-0.580390	134	Secondary calcareous grassland meadows and scrub
Totternhoe Quarry	51.891990	-0.568360	125	Secondary calcareous grassland meadows and scrub
Winterbourne Downs	51.149630	-1.685000	114	Secondary calcareous grassland meadows and scrub

NEOTROPICAL Panama				
Barro Colorado Island	9.164550	-79.837554	40	Secondary semi-deciduous lowland tropical wet forest
El Valle	8.617975	-80.115683	735	Lowland tropical wet encroaching scrub
Gamboa	9.116637	-79.696492	50	Lowland managed urban green spaces
Mount Totumas	8.882948	-82.683699	1880	Lower mountain rain forest and managed agroforestry sites
Parque Nacional Altos de Campana	8.705518	-79.887839	284	Premontane wet encroaching scrub and secondary forest
Parque Nacional San Lorenzo	9.246993	-79.978955	177	Secondary lowland tropical wet forest
Parque Nacional Soberanía	9.125578	-79.714579	84	Secondary semi-deciduous lowland tropical wet forest
Sajalices	8.680064	-79.880754	98	Lowland tropical wet encroaching scrub and secondary forest

SUPPLEMENTARY TABLE 2.2. *Sources of photos used to calculate wing aspect ratio.*

Name of organisation	Website
Butterflies of America	www.butterfliesofamerica.com
Natural History Museum Entomology collection	www.nhm.ac.uk/our-science/collections/entomology-collections.html
BOLD systems	www.boldsystems.org
MCZbase, Museum of Comparative Zoology, Harvard University	https://mczbase.mcz.harvard.edu
FUNDus, Universität Hamburg	https://fundus.uni-hamburg.de
Mapování motýlů ČR	www.lepidoptera.cz
Finnish Biodiversity Information Facility	https://laji.fi
Farfalle Italiane	www.farfalleitalia.it
Svenska fjärilar	www3.nrm.se/en/svenska_fjarilar/l/lepidoptera_family.html
Biological Library	www.biolib.cz



SUPPLEMENTARY PANEL 2.1. *Pearson correlation coefficient between predictor variables used for the linear mixed effect model in analysis 2: air temperature (T_a), mean forewing length (mm), mean wing aspect ratio and wing colour.*

SUPPLEMENTARY TABLE 2.3. *Results of one-way ANOVAs with mean forewing length (mm), mean wing aspect ratio and colour fitted separately as the response variable and family as the predictor variable. Statistically significant p -values are marked with an asterisk.*

	df	F	p
Mean forewing length	5	891	<0.001*
Mean wing aspect ratio	5	149	<0.001*
Colour	5	6633	<0.001*

SUPPLEMENTARY TABLE 2.4. *Temperate and neotropical species, with their taxonomic family, mean forewing length (mm), mean forewing aspect ratio, wing colour, the sample size of individuals we obtained in the field, buffering ability estimate (calculated as the slope of the regression of thoracic temperature against air temperature, subtracted from 1, following Bladon et al 2020) and whether or not we have ≥ 10 microclimate temperature data points per species. If ≥ 10 microclimate temperature data points were available for a species, this species was included in the analysis of microclimate selection, postural thermoregulation and index of thermal specialisation.*

Species	Family	Forewing length mm	Forewing aspect ratio	Wing colour	Sample size	Buffering ability estimate	Microclimate data
TEMPERATE							
<i>Aglais io</i>	Nymphalidae	28.479	1.635	4	83	0.362	Y
<i>Aglais urticae</i>	Nymphalidae	24.618	1.727	3	42	0.295	Y
<i>Anthocharis cardamines</i>	Pieridae	21.124	1.776	2	54	0.601	N
<i>Aphantopus hyperantus</i>	Nymphalidae	21.550	1.631	5	353	0.212	Y
<i>Araschnia levana</i>	Nymphalidae	17.500	1.858	5	124	0.164	Y
<i>Argynnis paphia</i>	Nymphalidae	32.000	1.785	3	33	0.223	Y
<i>Aricia agestis</i>	Lycaenidae	13.246	1.770	4	152	0.191	Y
<i>Carterocephalus palaemon</i>	Hesperiidae	14.000	2.069	4	10	0.537	Y
<i>Celastrina argiolus</i>	Lycaenidae	15.675	1.601	3.5	24	0.311	N
<i>Coenonympha pamphilus</i>	Nymphalidae	15.063	1.641	4	330	0.133	N
<i>Colias hyale</i>	Pieridae	25.000	1.754	2	15	0.080	N
<i>Cupido minimus</i>	Lycaenidae	11.838	1.696	4	128	0.189	N
<i>Erebia aethiops</i>	Nymphalidae	23.000	1.741	4	115	0.290	Y
<i>Erebia epiphron</i>	Nymphalidae	16.362	1.831	4	239	-0.100	Y
<i>Erebia euryale</i>	Nymphalidae	21.500	1.688	4	59	0.218	Y
<i>Erebia ligea</i>	Nymphalidae	25.500	1.694	4	175	0.415	Y
<i>Erebia medusa</i>	Nymphalidae	21.500	2.017	4	22	0.675	N
<i>Erebia melampus</i>	Nymphalidae	14.000	1.679	4	19	0.214	N
<i>Erebia pandrose</i>	Nymphalidae	21.000	1.764	4	43	0.472	N
<i>Erynnis tages</i>	Hesperiidae	14.592	1.738	5	78	0.213	N
<i>Gonepteryx rhamni</i>	Pieridae	29.279	1.598	1.5	120	0.393	Y
<i>Hamearis lucina</i>	Riodinidae	15.062	1.726	4	40	-0.404	N
<i>Leptidea sinapis</i>	Pieridae	21.500	2.017	1	74	0.190	N

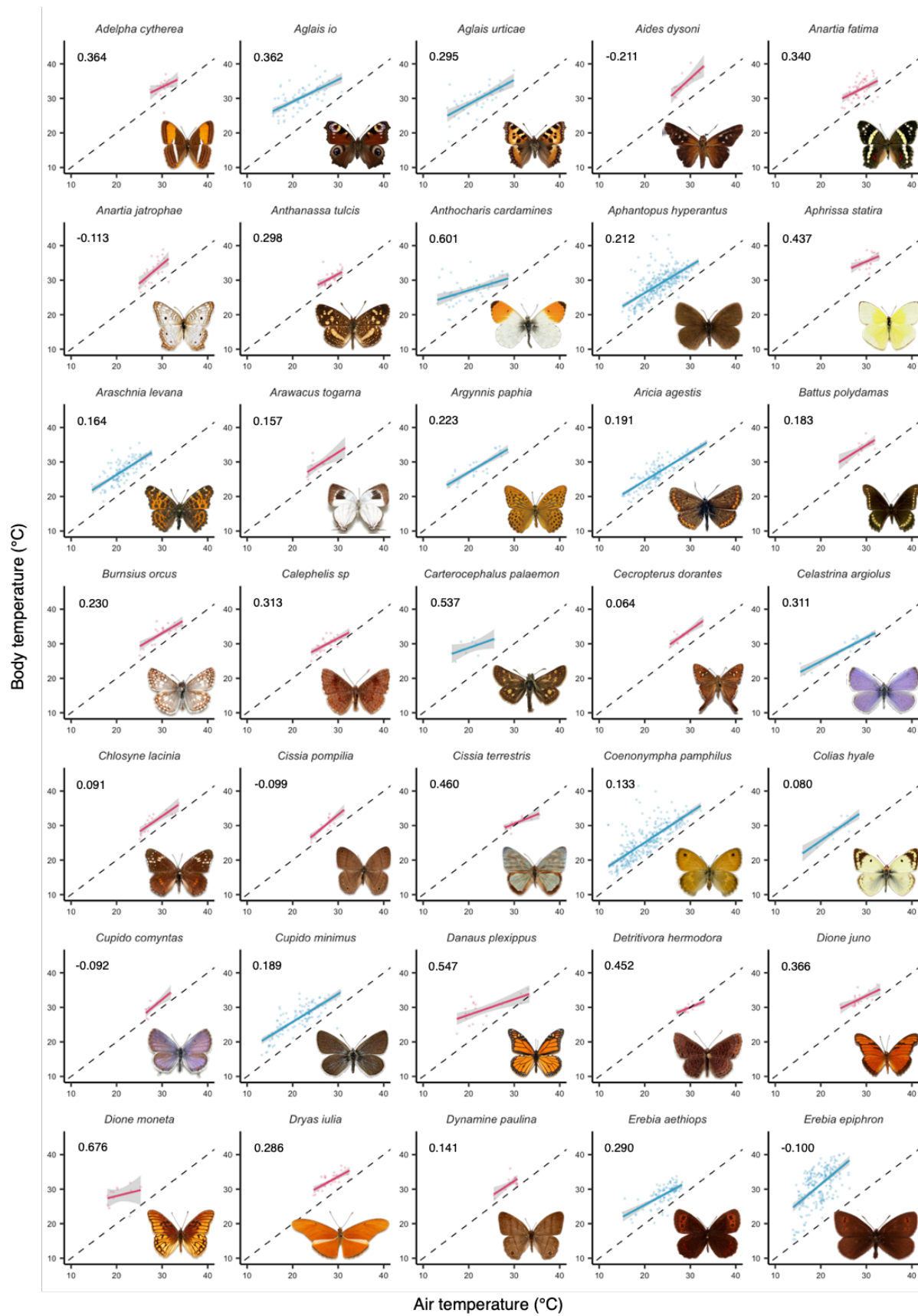
<i>Lycaena phlaeas</i>	Lycaenidae	14.717	1.699	4	50	0.128	Y
<i>Lysandra coridon</i>	Lycaenidae	17.084	1.699	4	211	0.221	Y
<i>Maniola jurtina</i>	Nymphalidae	22.665	1.553	4	467	0.201	Y
<i>Melanargia galathea</i>	Nymphalidae	24.382	1.662	3.5	267	0.120	Y
<i>Ochlodes sylvanus</i>	Hesperiidae	15.748	1.873	4	141	0.455	Y
<i>Pararge aegeria</i>	Nymphalidae	21.478	1.643	4	173	0.195	Y
<i>Pieris brassicae</i>	Pieridae	30.494	1.768	1	128	0.370	Y
<i>Pieris napi</i>	Pieridae	23.502	1.730	1	311	0.273	Y
<i>Pieris rapae</i>	Pieridae	23.943	1.743	1	278	0.317	Y
<i>Polygonia c-album</i>	Nymphalidae	23.580	1.647	4	42	0.135	Y
<i>Polyommatus icarus</i>	Lycaenidae	14.422	1.718	4	640	0.192	Y
<i>Pyronia tithonus</i>	Nymphalidae	18.993	1.592	4	141	0.103	Y
<i>Speyeria aglaja</i>	Nymphalidae	28.944	1.652	3	20	0.040	N
<i>Thymelicus lineola</i>	Hesperiidae	12.811	1.799	4	72	0.334	Y
<i>Thymelicus sylvestris</i>	Hesperiidae	13.839	1.884	4	153	0.135	Y
<i>Vanessa atalanta</i>	Nymphalidae	30.270	1.643	4.5	20	0.487	N
<i>Vanessa cardui</i>	Nymphalidae	29.161	1.769	4	36	0.155	N
NEOTROPICAL							
<i>Adelpha cytherea</i>	Nymphalidae	24.335	1.551	4	24	0.364	N
<i>Aides dysoni</i>	Hesperiidae	24.814	2.233	5	14	-0.211	N
<i>Anartia fatima</i>	Nymphalidae	28.902	1.658	5	63	0.340	Y
<i>Anartia jatrophae</i>	Nymphalidae	28.705	1.729	3.5	27	-0.113	N
<i>Anthanassa tulcis</i>	Nymphalidae	15.503	1.820	5	30	0.298	Y
<i>Aphrissa statira</i>	Pieridae	32.204	1.584	2	24	0.437	N
<i>Arawacus togarna</i>	Lycaenidae	14.492	1.463	3.5	13	0.157	N
<i>Battus polydamas</i>	Papilionidae	43.615	1.900	6	15	0.183	N
<i>Burnsius orcus</i>	Hesperiidae	14.648	1.848	3.5	26	0.230	Y
<i>Calephelis sp</i>	Riodinidae	11.056	1.575	4	27	0.313	Y
<i>Cecropterus dorantes</i>	Hesperiidae	23.392	1.804	5	13	0.064	N
<i>Chlosyne lacinia</i>	Nymphalidae	22.347	1.847	4.5	16	0.091	N
<i>Cissia pompilia</i>	Nymphalidae	18.264	1.641	5	18	-0.099	Y
<i>Cissia terrestris</i>	Nymphalidae	15.592	1.572	5	13	0.460	N

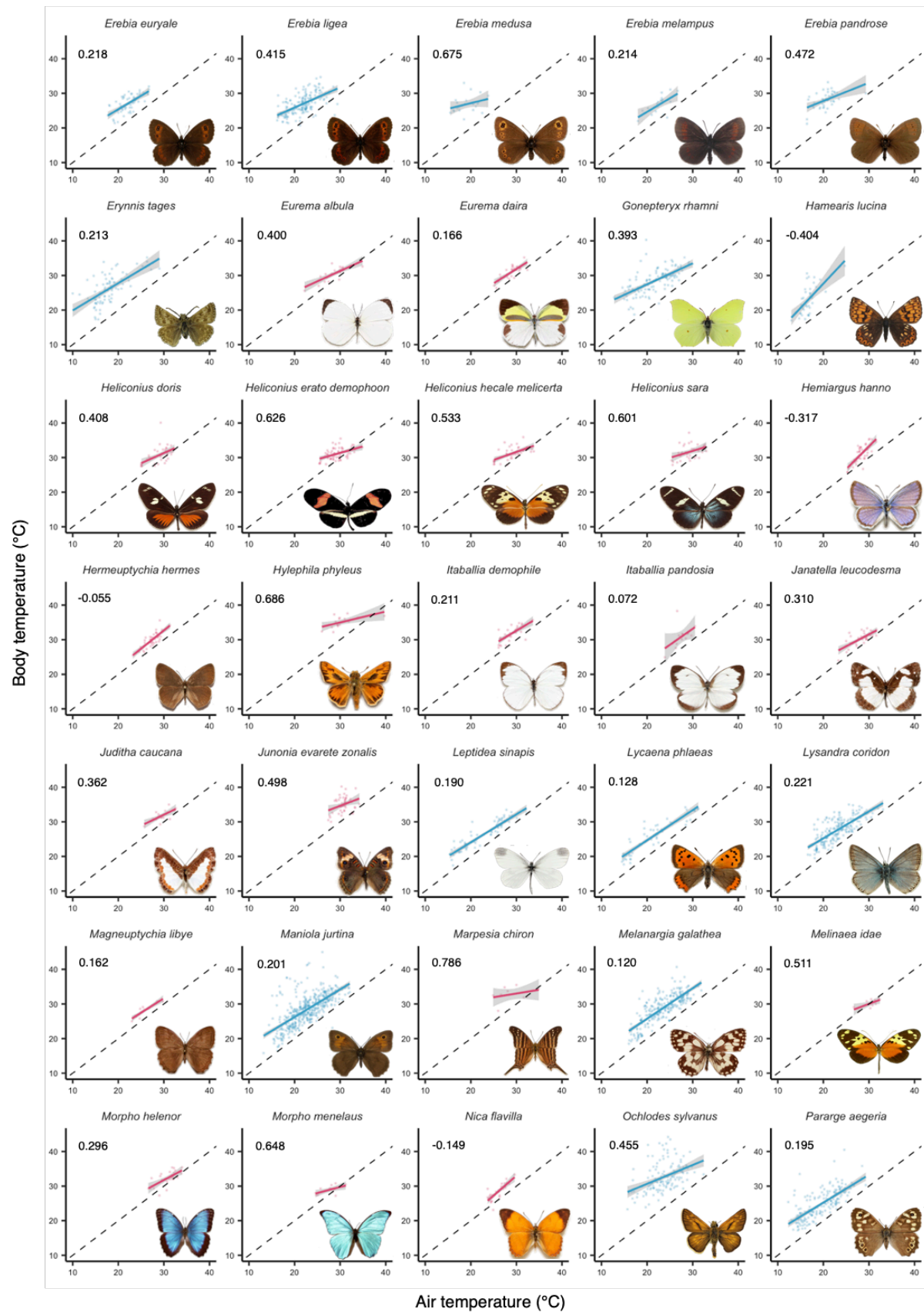
<i>Cupido comyntas</i>	Lycaenidae	10.200	1.615	4	12	-0.092	N
<i>Danaus plexippus</i>	Nymphalidae	47.315	1.966	4.5	22	0.547	N
<i>Detritivora hermodora</i>	Riodinidae	11.211	1.608	5	14	0.452	N
<i>Dione juno</i>	Nymphalidae	37.611	2.090	3	20	0.366	N
<i>Dione moneta</i>	Nymphalidae	36.991	2.013	3	12	0.676	N
<i>Dryas iulia</i>	Nymphalidae	41.658	2.471	3	36	0.286	N
<i>Dynamine paulina</i>	Nymphalidae	21.453	1.581	4	17	0.141	N
<i>Eurema albula</i>	Pieridae	18.411	1.549	1	22	0.400	N
<i>Eurema daira</i>	Pieridae	16.092	1.939	1.5	28	0.166	N
<i>Heliconius doris</i>	Nymphalidae	39.581	2.270	6	42	0.408	N
<i>Heliconius erato demophoon</i>	Nymphalidae	33.534	1.972	6	65	0.626	N
<i>Heliconius hecale melicerta</i>	Nymphalidae	42.244	2.082	4.5	44	0.533	N
<i>Heliconius sara</i>	Nymphalidae	32.750	2.260	6	35	0.601	N
<i>Hemiargus hanno</i>	Lycaenidae	9.169	1.692	4	42	-0.317	N
<i>Hermeuptychia hermes</i>	Nymphalidae	16.091	1.497	5	47	-0.055	Y
<i>Hylephila phyleus</i>	Hesperiidae	14.823	2.000	4	16	0.686	N
<i>Itaballia demophile</i>	Pieridae	28.091	1.637	1	34	0.211	N
<i>Itaballia pandosia</i>	Pieridae	21.062	1.648	1	13	0.072	N
<i>Janatella leucodesma</i>	Nymphalidae	18.343	1.712	3.5	35	0.310	Y
<i>Juditha caucana</i>	Riodinidae	15.768	1.642	3.5	18	0.362	N
<i>Junonia evarete zonalis</i>	Nymphalidae	26.759	1.584	4	50	0.498	Y
<i>Magneuptychia libye</i>	Nymphalidae	24.783	1.532	5	13	0.162	N
<i>Marpesia chiron</i>	Nymphalidae	30.094	1.792	5	10	0.786	N
<i>Melinaea idae</i>	Nymphalidae	43.445	2.109	4.5	10	0.511	N
<i>Morpho helenor</i>	Nymphalidae	58.428	1.593	3	25	0.296	N
<i>Morpho menelaus</i>	Nymphalidae	68.612	1.763	3	13	0.648	N
<i>Nica flavilla</i>	Nymphalidae	20.523	1.594	4	28	-0.149	Y
<i>Pareuptychia ocirrhoe</i>	Nymphalidae	18.896	1.549	3.5	12	0.030	N
<i>Parides eurimedes</i>	Papilionidae	38.662	1.887	6	13	0.553	N
<i>Parides sesostris</i>	Papilionidae	39.950	1.873	6	24	0.367	N
<i>Phoebis argante</i>	Pieridae	33.340	1.473	2	39	1.005	N
<i>Phoebis philea</i>	Pieridae	38.473	1.497	2	12	0.337	N

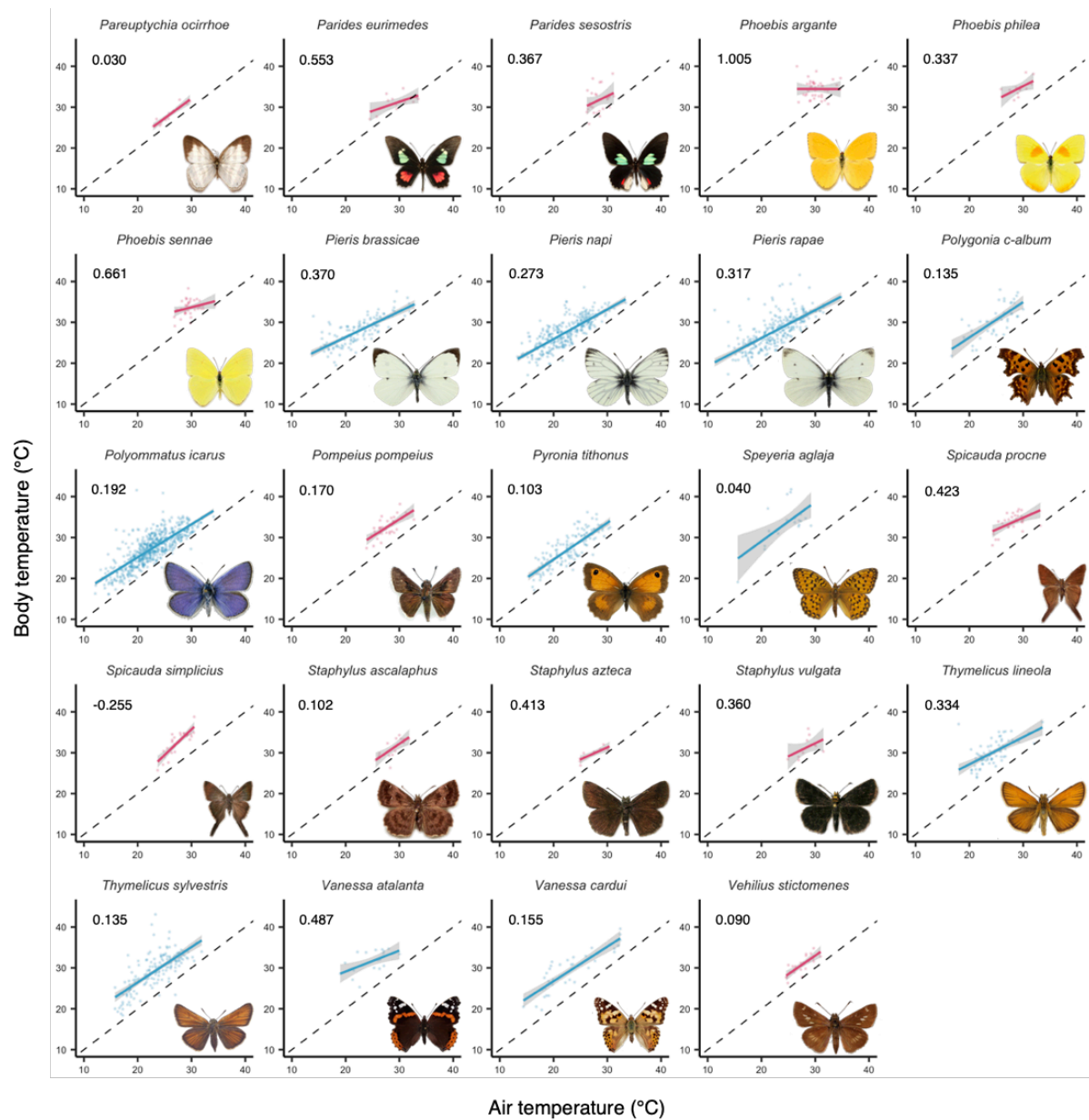
<i>Phoebis sennae</i>	Pieridae	33.151	1.518	2	37	0.661	N
<i>Pompeius pompeius</i>	Hesperiidae	14.631	1.835	5	36	0.170	N
<i>Spicauda procne</i>	Hesperiidae	22.182	1.822	5	29	0.423	Y
<i>Spicauda simplici</i>	Hesperiidae	22.284	1.654	5	27	-0.255	N
<i>Staphylus ascalaphus</i>	Hesperiidae	13.008	1.727	5.5	15	0.102	Y
<i>Staphylus azteca</i>	Hesperiidae	12.760	1.768	5	10	0.413	N
<i>Staphylus vulgata</i>	Hesperiidae	12.950	1.765	5	12	0.360	Y
<i>Vehilius stictomenes</i>	Hesperiidae	11.425	1.902	5	21	0.090	Y

SUPPLEMENTARY RESULTS 2.1. *Summary statistics of the three temperate regions.*

In the UK, 1,884 individuals were measured across 29 species and five families (Hesperiidae, Lycaenidae, Nymphalidae, Pieridae and Riodinidae). T_a ranged from 10.0 °C to 34.8 °C, with a mean of 22.3 °C. T_b ranged from 15.4 °C to 45.0 °C, with a mean of 28.1 °C. In Austria, 383 individuals were measured across six species and one family (Nymphalidae). T_a ranged from 16.2 °C to 29.4 °C, with a mean of 22.4 °C. T_b ranged from 20.7 °C to 34.7 °C, with a mean of 27.5 °C. In the Czech Republic 1,302 individuals were measured across 33 species and four families (Hesperiidae, Lycaenidae, Nymphalidae and Pieridae). T_a ranged from 14.4 °C to 32.2 °C, with a mean of 22.0 °C. T_b ranged from 16.5 °C to 39.4 °C, with a mean of 27.8 °C.







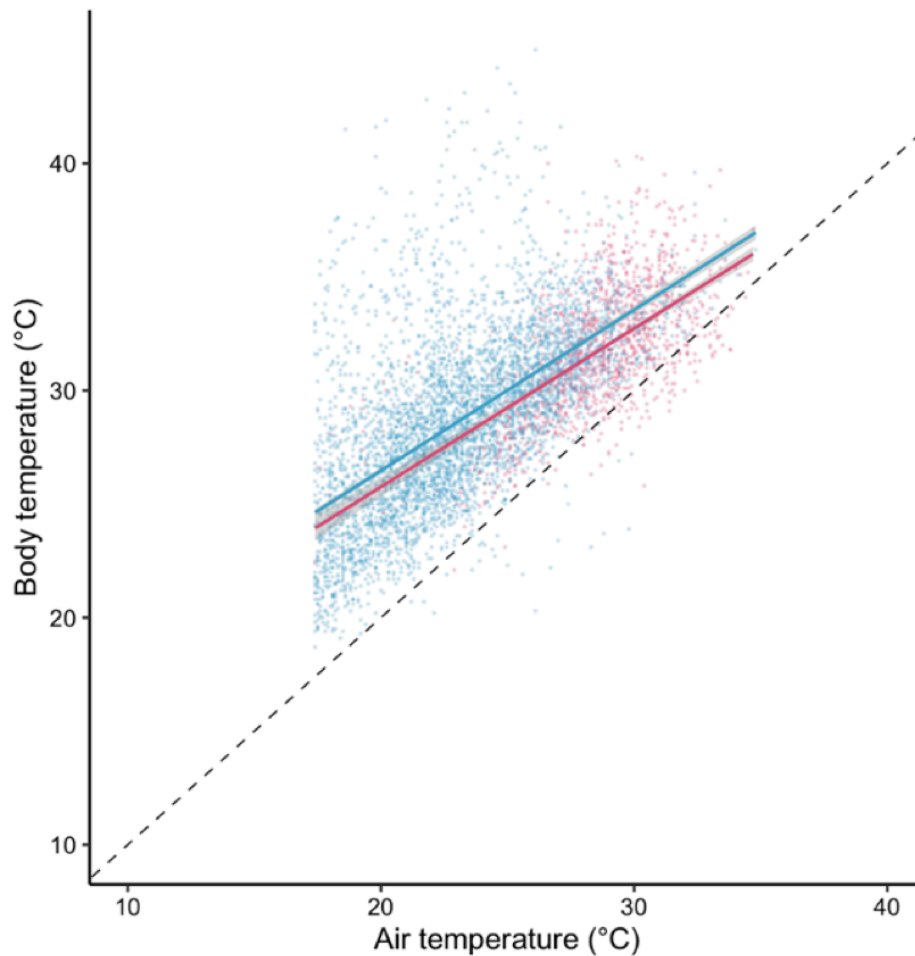
SUPPLEMENTARY FIGURE 2.2. Individual butterfly body temperatures (°C) at different air temperatures (°C) from neotropical (pink) and temperate (blue) regions for each species. Solid lines represent the modelled relationship between body temperature and air temperature. The grey bands show the 95% confidence intervals. The black dashed line represents a 1:1 relationship between body and air temperature. The buffering ability estimate is in the top left corner and is calculated as the slope of a simple linear regression, with body temperature as the response variable and air temperature as the predictor variable, subtracted from one. See Supplementary Table 2.5 for inset photo credits. Photos are not to scale.

SUPPLEMENTARY TABLE 2.5. *Photo credits for inset photos in Supplementary Fig. 2.2*

Species	Photo credit	License
TEMPERATE		
<i>Aglais io</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Aglais urticae</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Anthocharis cardamines</i>	Pekka Malinen	CC BY-SA 4.0
<i>Aphantopus hyperantus</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Araschnia levana</i>	Eino Ylönen	CC BY-SA 4.0
<i>Argynnis paphia</i>	Eino Ylönen	CC BY-SA 4.0
<i>Aricia agestis</i>	Bartoňová, Beneš, Faltýnek, Konvička. https://doi.org/10.3897/nl.42.38853.figure2	CC BY 4.0
<i>Carterocephalus palaemon</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Celastrina argiolus</i>	Jani Järvi, Jani Järvi	CC BY-SA 4.0
<i>Coenonympha pamphilus</i>	Pekka Malinen	CC BY-SA 4.0
<i>Colias hyale</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Cupido minimus</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Erebia aethiops</i>	M. Fatahi	All rights reserved - please ask for the terms of use if required
<i>Erebia epiphron</i>	M. Fatahi	All rights reserved - please ask for the terms of use if required
<i>Erebia euryale</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Erebia ligea</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Erebia medusa</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Erebia melampus</i>		CC BY-SA 4.0
<i>Erebia pandrose</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Erynnis tages</i>	Natural History Museum (2014). Specimens (from Collection n specimens) [BNHH (E) 1338624]	© Images copyright Trustees Natural History Museum, used with permission
<i>Gonepteryx rhamni</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Hamearis lucina</i>	Natural History Museum (2014). Specimens (from Collection n specimens) [BNHM (E) 1123540]	© Images copyright Trustees Natural History Museum, used with permission
<i>Leptidea sinapis</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Lycaena phlaeas</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Lysandra coridon</i>	M. Fatahi	All rights reserved - please ask for the terms of use if required
<i>Maniola jurtina</i>	Pekka Malinen	CC BY-SA 4.0
<i>Melanargia galathea</i>	M. Fatahi	All rights reserved - please ask for the terms of use if required
<i>Ochlodes sylvanus</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Pararge aegeria</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Pieris brassicae</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Pieris napi</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Pieris rapae</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Polygonia c-album</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Polyommatus icarus</i>	Pekka Malinen	CC BY-SA 4.0
<i>Pyronia tithonus</i>	Katharina Schneeberg	All rights reserved - please ask for the terms of use if required. Specimen from the collection of POLLICHIA
<i>Speyeria aglaja</i>	Eino Ylönen	CC BY-SA 4.0
<i>Thymelicus lineola</i>	Pekka Malinen	CC BY-SA 4.0
<i>Thymelicus sylvestris</i>	Natural History Museum (2014). Specimens (from Collection n specimens) [BNHM (E) 1357509]	© Images copyright Trustees Natural History Museum, used with permission
<i>Vanessa atalanta</i>	J. Tyllinen	CC BY-SA 4.0
<i>Vanessa cardui</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0

NEOTROPICAL		
<i>Adelpha cytherea</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Aides dysoni</i>	Bernard Hermier	© Images copyright Trustees Natural History Museum, used with permission
<i>Anartia fatima</i>	D.H. Janzen, W. Hallwachs 2011	CC BY-SA 4.0
<i>Anartia jatrophae</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Anthanassa tulcis</i>	Jim P. Brock 2009	CC BY-SA 4.0
<i>Aphrissa statira</i>	Andrew D. Warren 2011	CC BY-SA 4.0
<i>Arawacus togarna</i>	Kim Davis, Mike Strangeland, Andrew Warren 2008	CC BY-SA 4.0
<i>Battus polydamas</i>	Kim Davis, Mike Strangeland 2005	CC BY-SA 4.0
<i>Burnsius orcus</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Calephelis sp</i>	Kim Davis, Mike Strangeland, Andrew Warren 2008	CC BY-SA 4.0
<i>Cecropterus dorantes</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Chlosyne lacinia</i>	Kim Davis, Mike Strangeland 2006	CC BY-SA 4.0
<i>Cissia pompilia</i>	Kim Davis, Mike Strangeland 2006	CC BY-SA 4.0
<i>Cissia terrestris</i>	Nick V. Grishin 2010	CC BY-SA 4.0
<i>Cupido comyntas</i>	Kim Davis, Mike Strangeland, Andrew Warren 2010	CC BY-SA 4.0
<i>Danaus plexippus</i>	Andrew Warren 2011	CC BY-SA 4.0
<i>Detritivora hermodora</i>	Kim Davis, Mike Strangeland, Andrew Warren 2008	CC BY-SA 4.0
<i>Dione juno</i>	Kim Davis, Mike Strangeland 2005	CC BY-SA 4.0
<i>Dione moneta</i>	Andrew Warren 2010	CC BY-SA 4.0
<i>Dryas iulia</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Dynamine paulina</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Eurema albula</i>	Andrew D. Warren 2011	CC BY-SA 4.0
<i>Eurema daira</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Heliconius doris</i>	Kim Davis, Mike Strangeland, Andrew Warren 2010	CC BY-SA 4.0
<i>Heliconius erato</i>	Gerardo Lamas	© Images copyright Trustees Natural History Museum, used with permission
<i>Heliconius hecale</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Heliconius sara</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Hemiargus hanno</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Hermeuptychia hermes</i>	Kim Davis, Mike Strangeland 2006	CC BY-SA 4.0
<i>Hylephila phyleus</i>	Jim P. Brock 2009	CC BY-SA 4.0
<i>Itaballia demophile</i>	Kim Davis, Mike Strangeland, Andrew Warren 2010	CC BY-SA 4.0
<i>Itaballia pandosia</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Janatella leucodesma</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Juditha caucana</i>	Kim Davis, Mike Strangeland, Andrew Warren 2010	CC BY-SA 4.0
<i>Junonia zonalis</i>	John Calhoun 2010	CC BY-SA 4.0
<i>Magneuptychia libye</i>	Jim P. Brock 2011	CC BY-SA 4.0
<i>Marpesia chiron</i>	Andrew Warren 2011	CC BY-SA 4.0

<i>Melinaea idae</i>	Keith Willmott 2010	CC BY-SA 4.0
<i>Morpho helenor</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Morpho menelaus</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Nica flavilla</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Pareuptychia ocirrhoe</i>	Kim Davis, Mike Strangeland 2010	CC BY-SA 4.0
<i>Parides eurimedes</i>	Jim P. Brock 2009	CC BY-SA 4.0
<i>Parides sesostris</i>	Kim Davis, Mike Strangeland, Andrew Warren 2008	CC BY-SA 4.0
<i>Phoebis argante</i>	Andrew D. Warren 2011	CC BY-SA 4.0
<i>Phoebis philea</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Phoebis sennae</i>	Andrew D. Warren 2011	CC BY-SA 4.0
<i>Pompeius pompeius</i>	Andrew D. Warren 2007	CC BY-SA 4.0
<i>Spicauda procne</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Spicauda simplicius</i>	Nick V. Grishin 2013	CC BY-SA 4.0
<i>Staphylus ascalaphus</i>	Kim Davis, Mike Strangeland, Andrew Warren 2009	CC BY-SA 4.0
<i>Staphylus azteca</i>	D.H. Janzen, W. Hallwachs 2010	CC BY-SA 4.0
<i>Staphylus vulgata</i>	D.H. Janzen, W. Hallwachs 2010	CC BY-SA 4.0
<i>Vehilius stictomenes</i>	Bernard Hermier	CC BY-SA 4.0



SUPPLEMENTARY FIGURE 2.3. *Individual butterfly body temperatures (°C) at different air temperatures (°C) from neotropical (pink) and temperate (blue) regions, restricted to only include air temperatures within the range experienced in both regions. Solid lines represent the modelled relationship between body temperature and air temperature. The grey bands show the 95% confidence intervals. The black dashed line represents a 1:1 relationship between body and air temperature.*

SUPPLEMENTARY RESULTS 2.2. *Buffering ability - controlling for differences in air temperature between neotropical and temperate regions across the full assemblage.*

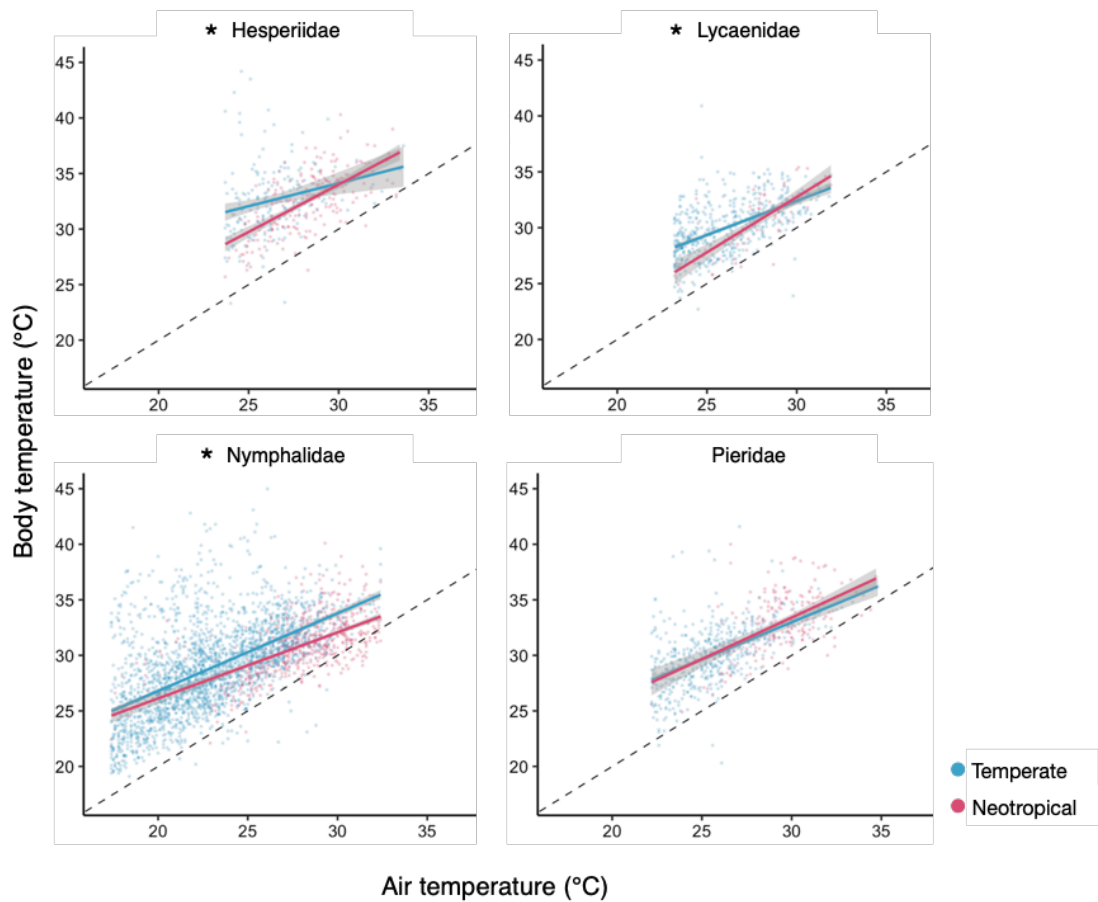
When the data were restricted to only include T_a within the range experienced in both regions, the mean buffering estimate was higher for neotropical butterflies than temperate butterflies (neotropical butterflies: 0.331 ± 0.034 ; temperate butterflies 0.249 ± 0.011 ; $\chi^2 = 5.673$, $df = 1$, $p = 0.017$; Supplementary Fig. 2.3).

SUPPLEMENTARY TABLE 2.6. *Buffering ability estimates, standard error (SE) and number of species (n) per family for temperate and neotropical families. Results of the likelihood ratio test between models with and without the two-way interaction between region and air temperature are given (test-statistic (χ^2), degrees of freedom (df) and likelihood p-value). Statistically significant likelihood p values are marked with an asterisk and indicate a significant difference in buffering ability between neotropical and temperate regions for species in that family.*

Family	Temperate			Neotropical			χ^2	df	Likelihood p-value
	Buffering ability estimate	SE	n	Buffering ability estimate	SE	n			
Hesperiidae	0.269	0.039	5	0.212	0.099	11	0.346	1	0.557
Lycaenidae	0.197	0.017	6	-0.020	0.149	3	2.021	1	0.155
Nymphalidae	0.180	0.015	21	0.397	0.047	26	21.663	1	< 0.001*
Pieridae	0.326	0.019	7	0.426	0.092	8	1.101	1	0.294
Riodinidae	-0.404	0.181	1	0.373	0.233	3	11.831	1	< 0.001*

SUPPLEMENTARY TABLE 2.7. *Buffering ability estimates, standard error (SE) and number of species (n) per family for temperate and tropical families. To control for differences in air temperature between neotropical and temperate regions, data were restricted to the air temperature range experienced in both tropical and temperate regions. Results of the likelihood ratio test between models with and without the two-way interaction between region and air temperature are given (test-statistic (χ^2), degrees of freedom (df) and likelihood p-value). Statistically significant p values are marked with an asterisk and indicate a significant difference in buffering ability between neotropical and temperate regions for species in that family.*

Family	Temperate			Neotropical			χ^2	df	Likelihood p-value
	Buffering ability estimate	SE	n	Buffering ability estimate	SE	n			
Hesperiidae	0.588	0.092	3	0.134	0.122	11	13.765	1	< 0.001*
Lycaenidae	0.390	0.038	6	0.011	0.129	3	8.594	1	0.003*
Nymphalidae	0.221	0.017	21	0.361	0.052	23	7.393	1	0.007*
Pieridae	0.301	0.043	7	0.415	0.091	8	1.449	1	0.229



SUPPLEMENTARY FIGURE 2.4. Individual butterfly body temperatures ($^{\circ}\text{C}$) at different air temperatures ($^{\circ}\text{C}$) from neotropical (pink) and temperate (blue) regions for each taxonomic family, restricted to only include air temperatures within the range experienced in both regions. Solid lines represent the modelled relationship between body temperature and air temperature. The grey bands show the 95% confidence intervals. The black dashed line represents a 1:1 relationship between body and air temperature. Families with a significant difference in buffering ability estimate between neotropical and temperate species are marked with an asterisk.

SUPPLEMENTARY RESULTS 2.3. Buffering ability - controlling for differences in air temperature between neotropical and temperate regions by family.

Temperate Hesperidae and temperate Lycaenidae were better at buffering their T_b against changes in T_a than Hesperidae and Lycaenidae from neotropical regions (Hesperidae: $\chi^2 = 13.765$, $df = 1$, $p < 0.001$; Lycaenidae: $\chi^2 = 8.594$, $df = 1$, $p = 0.003$) (Supplementary Fig. 2.4, Supplementary Table 2.7). Neotropical Nymphalidae were better at buffering their T_b against changes in T_a than Nymphalidae from temperate regions ($\chi^2 = 7.393$, $df = 1$, $p = 0.007$) (Supplementary Fig. 2.4, Supplementary Table 2.7). There was no difference in the buffering

ability of Pieridae from neotropical and temperate regions ($\chi^2 = 1.445$, $df = 1$, $p = 0.229$). There were insufficient data on Riodinidae species to undertake this analysis.

SUPPLEMENTARY TABLE 2.8. *Output from the best fitting linear mixed effects model, with body temperature (T_b) as the predictor variable and air temperature (T_a), region (neotropical and temperate), mean forewing length, mean forewing aspect ratio and the two-way interactions between T_a and each of mean forewing length and mean forewing aspect ratio as response variables. Data were restricted to the air temperature range experienced in both tropical and temperate regions. Mean forewing length was $\log_{10}$ transformed to ensure it was on the same scale as other predictor variables. Species was included as a random effect. Significant terms are marked with an asterisk.*

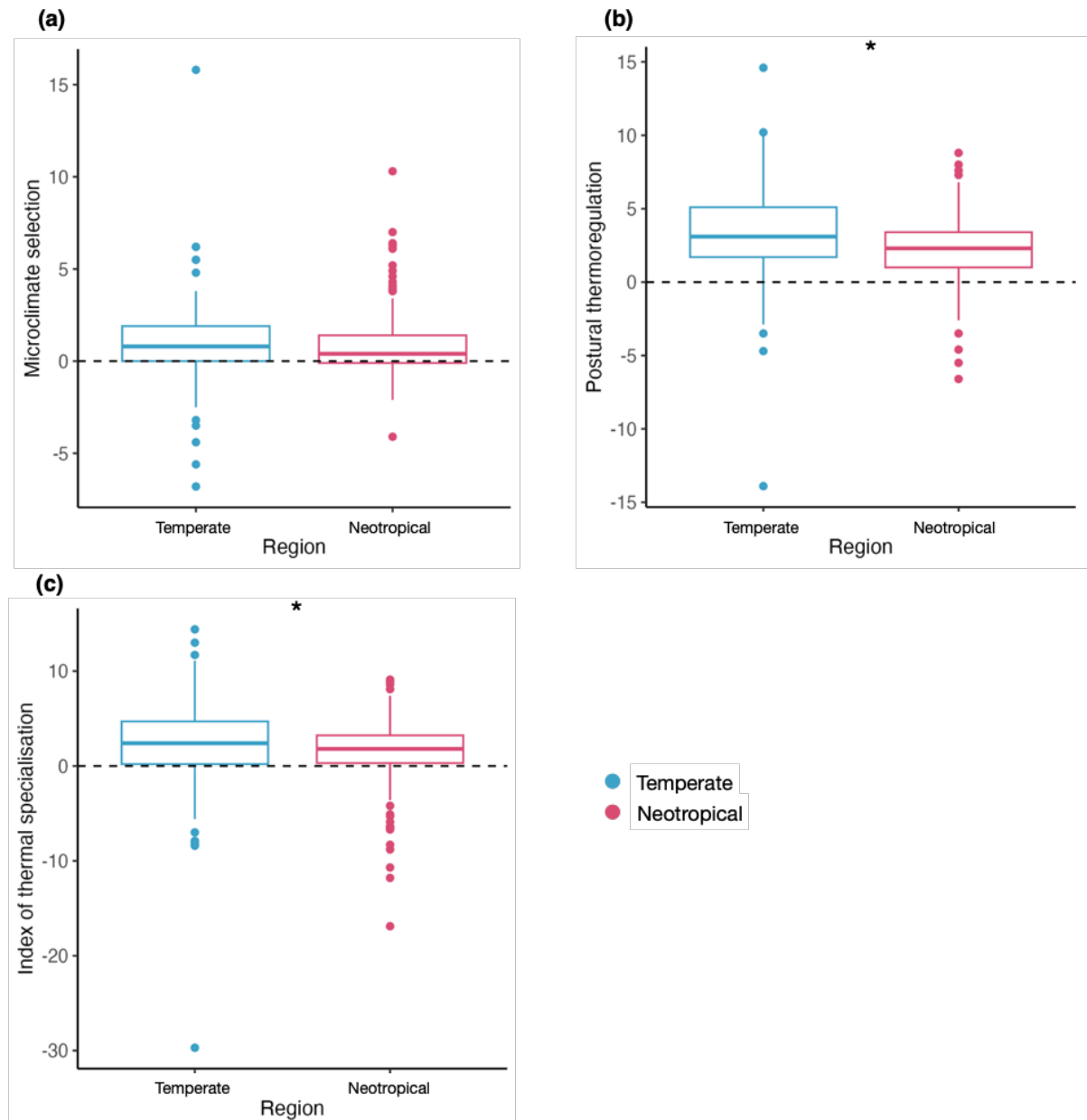
	Estimate	SE	df	F	<i>p</i>
Region: temperate	-11.643	4.947	1	18.390	<0.001*
Region: tropical	-10.264	0.322			
T_a	1.491	0.168	1	78.403	<0.001*
Mean forewing length	3.140	1.012	1	9.623	0.002*
Mean forewing aspect ratio	8.129	2.452	1	10.991	<0.001*
T_a : Mean forewing length	-0.212	0.079	1	7.258	0.007*
T_a : Mean forewing aspect ratio	-0.274	0.083	1	11.000	<0.001*

SUPPLEMENTARY TABLE 2.9. Mean values for microclimate selection, postural thermoregulation and index of thermal specialisation, the corresponding standard errors (SE) and number of species (n) across the whole assemblage and per family for temperate and neotropical regions. Results of the likelihood ratio test between models with and without the two-way region:air temperature interaction, and the region term, are given (test-statistic (χ^2), degrees of freedom (df) and likelihood p-value). Statistically significant p-values are marked with an asterisk and indicate a significant difference in the use of microclimate selection or postural thermoregulation, or in the index of thermal specialisation, between neotropical and temperate regions for species across the assemblage or within one family. In these cases, the higher value is marked in bold.

Microclimate selection												
Group	Temperate			Neotropical			Region:air temperature interaction			Region + air temperature		
	$\bar{x}$	SE	n	$\bar{x}$	SE	n	χ^2	df	Likelihood p-value	χ^2	df	Likelihood p-value
Assemblage	1.298	0.070	26	0.846	0.135	13	0.491	1	0.484	0.104	1	0.747
Hesperiidae	1.329	0.234	3	0.674	0.229	5	1.890	1	0.169	0.025	1	0.875
Nymphalidae	1.400	0.105	15	0.879	0.178	7	0.283	1	0.595	0.007	1	0.932
Postural thermoregulation												
Group	Temperate			Neotropical			Region:air temperature interaction			Region + air temperature		
	$\bar{x}$	SE	n	$\bar{x}$	SE	n	χ^2	df	Likelihood p-value	χ^2	df	Likelihood p-value
Assemblage	4.220	0.109	26	2.200	0.171	13	0.075	1	0.785	8.314	1	0.004*
Hesperiidae	6.259	0.525	3	2.726	0.357	5	0.239	1	0.625	2.431	1	0.119
Nymphalidae	4.723	0.151	15	2.103	0.200	7	0.601	1	0.438	11.855	1	<0.001*
Index of thermal specialisation												
Group	Temperate			Neotropical			Region:air temperature interaction			Region + air temperature		
	$\bar{x}$	SE	n	$\bar{x}$	SE	n	χ^2	df	Likelihood p-value	χ^2	df	Likelihood p-value
Assemblage	2.922	0.144	26	1.355	0.269	13	0.000	1	0.997	7.15	1	0.008*
Hesperiidae	4.930	0.531	3	2.053	0.523	5	0.059	1	0.809	2.516	1	0.113
Nymphalidae	3.323	0.210	15	1.224	0.328	7	0.037	1	0.848	7.97	1	0.005*

SUPPLEMENTARY RESULTS 2.4. *Microclimate selection, postural thermoregulation and ITS - controlling for differences in air temperature between neotropical and temperate regions.*

When data were restricted to the range of air temperatures which occurred in both regions, microclimate selection, postural thermoregulation and the ITS did not significantly differ between neotropical and temperate regions (microclimate selection: $\chi^2 = 0.001$, $df = 1$, $p = 0.972$; postural thermoregulation: $\chi^2 = 3.142$, $df = 1$, $p = 0.076$; ITS: $\chi^2 = 2.053$, $df = 1$, $p = 0.152$, Supplementary Fig. 2.5, Supplementary Table 2.10). This was the same for Nymphalidae when tested separately for microclimate selection ($\chi^2 = 0.085$, $df = 1$, $p = 0.771$) and ITS ($\chi^2 = 3.147$, $df = 1$, $p = 0.076$), but temperate Nymphalidae butterflies used postural thermoregulation to increase their temperature significantly more than neotropical butterflies ($\chi^2 = 6.445$, $df = 1$, $p = 0.011$, Supplementary Table 2.10). There was insufficient data to undertake this analysis on the other families.



SUPPLEMENTARY FIGURE 2.5. Microclimate selection (the difference between microclimate temperature and air temperature, a), postural thermoregulation (the difference between body temperature and microclimate temperature, b) and index of thermal specialisation (postural thermoregulation – microclimate selection, c) of temperate (blue) and neotropical (pink) butterflies. Data restricted to only include air temperatures within the range experienced in both regions. Significant differences between neotropical and temperate butterflies are marked with an asterisk.

SUPPLEMENTARY TABLE 2.10. Mean values for microclimate selection, postural thermoregulation and index of thermal specialisation, the corresponding standard errors (SE) and number of species (n) across the whole assemblage and per family for temperate and neotropical regions. Results of the likelihood ratio test between models with and without the two-way region:air temperature interaction, and the region term, are given (test-statistic (χ^2), degrees of freedom (df) and likelihood p-value). Statistically significant p-values are marked with an asterisk and indicate a significant difference in the use of microclimate selection or postural thermoregulation, or in the index of thermal specialisation, between neotropical and temperate regions for species across the assemblage or within one family. In these cases, the higher value is marked in bold. To control for differences in air temperature between neotropical and temperate regions, data were restricted to the air temperature range experienced in both neotropical and temperate regions.

Microclimate selection												
Group	Temperate			Neotropical			Region:air temperature interaction			Region + air temperature		
	$\bar{x}$	SE	n	$\bar{x}$	SE	n	χ^2	df	Likelihood p-value	χ^2	df	Likelihood p-value
Assemblage	0.943	0.122	14	0.846	0.135	13	1.292	1	0.256	0.001	1	0.972
Nymphalidae	0.969	0.223	7	0.879	0.178	7	0.103	1	0.749	0.085	1	0.771
Postural thermoregulation												
Group	Temperate			Neotropical			Region:air temperature interaction			Region + air temperature		
	$\bar{x}$	SE	n	$\bar{x}$	SE	n	χ^2	df	Likelihood p-value	χ^2	df	Likelihood p-value
Assemblage	3.216	0.179	14	2.200	0.171	13	1.223	1	0.269	3.142	1	0.076
Nymphalidae	3.845	0.310	7	2.103	0.200	7	3.243	1	0.072	6.445	1	0.011*
Index of thermal specialisation												
Group	Temperate			Neotropical			Region:air temperature interaction			Region + air temperature		
	$\bar{x}$	SE	n	$\bar{x}$	SE	n	χ^2	df	Likelihood p-value	χ^2	df	Likelihood p-value
Assemblage	2.273	0.264	14	1.355	0.269	13	0.090	1	0.764	2.053	1	0.152
Nymphalidae	2.876	0.488	7	1.224	0.327	7	0.757	1	0.384	3.147	1	0.076

Chapter 3

Regional differences in thermoregulation of European butterflies is driven by temperature, precipitation, and geographic region

Laird-Hopkins, B. C., Arnold, L., Ashe-Jepson, E., Cole, H., Eberhardt, L., Ewing, S., French-Constant, R. H., Freiberga, I., Hayes, M., Khalaf, A., Kleckova, I., Linke, D., Matos-Maraví, P., Martelli, F., McVeigh, A., Menendez, R., Rhodes, M., Talavera, G., Toro-Delgado, E., Turner, E. C., Vila, R., Sam, K., & Bladon, A. J. (*in review*). Regional differences in thermoregulation of European butterflies is driven by temperature, precipitation, and geographic region. *Communications Biology*.

3.1 Abstract

Climate change is causing species' distributional shifts and local extinctions. However, the effects of global change are not felt equally among species, and there is an urgent need to understand regional differences in species' thermal vulnerability. We compare the thermoregulatory ability of European butterfly populations to determine the drivers of regional differences. We found that Southern butterflies and those occupying warm temperate zones had the highest buffering ability, achieved by using both microclimate selection and postural thermoregulation. With increasing air temperatures, butterflies from dry and warm temperate zones become more reliant on microclimate selection for thermoregulation, putting them most at risk from rising temperatures, especially in homogenous anthropogenic landscapes. We also found intraspecific differences in the buffering ability of four species, following temperature, precipitation and geographic patterns. Thermoregulation in butterflies is a multifaceted response to geography and climate, so the impacts of climate change will be unequal across communities.

3.2 Introduction

Climate change is resulting in an increase in global temperatures and heatwave events, as well as changes in precipitation patterns (Lee et al., 2021). The effects of increasing ambient temperature and varying humidity on species can be direct, through associated alterations in their development, physiology, life-history, phenology and distribution, and indirect, via increased activities of parasites, parasitoids, competitors and diseases (Angilletta, 2009; Eggleton, 2020; Herrando et al., 2019; Parmesan, 2006; Sunday et al., 2014). Community-wide responses to climate change are thus expected, with knock-on effects through entire ecosystems (Parmesan, 2006). An increase in ambient temperature could result in an increased risk of desiccation, as high temperatures increase water stress, meaning that thermal performance depends on both ambient precipitation and temperature (Chown et al., 2011; Kleynhans & Terblanche, 2011; Klok & Chown, 1998). Therefore, understanding how species respond to changes in both ambient temperature and rainfall is pivotal to predict the impacts of climate change.

Butterflies are particularly sensitive to changing climatic conditions, and research into how they respond to changes in ambient temperature and rainfall is needed urgently (Diamond & Yilmaz, 2018; Harvey et al., 2020). Butterflies can modify their body temperature and water balance through physiological, morphological and behavioural means (Kemp & Krockenberger, 2002; Kleckova et al., 2014; Shanks et al., 2015). For example, insects can reduce their metabolic rate to avoid water loss, or produce cellular compounds which enhance desiccation tolerance (Chown et al., 2011). Additionally, morphology is important for determining thermoregulatory ability, with large butterflies better at buffering their body temperature against changes in ambient temperature than small butterflies (Bladon et al., 2020; Laird-Hopkins et al., 2023). Finally, behavioural modifications, such as microhabitat use and basking position and duration, can play an important role in thermoregulation and desiccation avoidance (Chown et al., 2011; Kearney et al., 2009; Kleckova et al., 2014; Tsai et al., 2020). Together, this allows individual butterflies to regulate their body temperature in response to the surrounding air temperature, a response known as thermal buffering ability (Bladon et al., 2020). Thermal buffering ability can vary both inter- and intra-specifically as a result of climatic differences in the local environment or as a consequence of adaptations or

phenotypic plasticity (Kleckova et al., 2014; Toro-Delgado et al., In review). However, the extent to which local climatic patterns, such as temperature and rainfall, drive these differences in buffering ability remains unknown.

Discerning the drivers of species' and population vulnerability to climate change can allow us to predict how communities will change under future environmental conditions (Diamond et al., 2011; Diamond & Yilmaz, 2018). Major differences in thermoregulation among populations are observed in species whose ranges cross different bioclimatic regions, altitudes and latitudes, where temperature and moisture supply differ greatly (Advani et al., 2019; Dongmo et al., 2021; Kleckova & Klecka, 2016; Montejo-Kovacevich et al., 2020). For example, high altitude butterfly populations are less tolerant of heat than low altitude populations, and tend to be smaller in size, which further reduces their thermoregulatory ability (Fischer & Karl, 2010; Wu et al., 2019). Ectotherms living in drier environments, such as in forest canopies, are more drought resistant than those living in the forest understory, where humidity is higher (Bujan et al., 2016). Local environmental conditions, including radiation levels and the availability of microclimates also affect species buffering ability. Hence, local populations seem to respond differently to specific changes in their environment, and this needs to be considered when predicting species' vulnerability to climate change.

A recent study using long-term monitoring data of 143 European butterfly species, across six bioclimatic regions found that butterfly population dynamics were driven by both local and global climate change indicating that these butterflies are adapted to their local climate (Melero et al., 2022). Furthermore, local adaptation was found to contain a strong phylogenetic signal. However, the mechanism underpinning regional differences in species trends remains unknown. To investigate this at a behavioural level, we compared the thermal buffering ability of 59 butterfly species across latitudes and bioclimatic regions in Europe, to test whether butterfly thermoregulatory ability and the mechanism (behavioural and/or microclimate selection) differs between species and populations depending on the local climate. We undertook our analysis within a phylogenetic framework to test whether there was a phylogenetic signal to any local differences detected. Specifically, we addressed the following questions:

1. Does buffering ability differ between butterfly assemblages from different geographic regions and climatic zones?
2. Does buffering ability differ between populations of individual species from different geographic regions and climatic zones?
3. Does the extent of thermoregulation via the use of microclimates or postural means, or the index of thermal specialisation, differ between butterfly assemblages from different geographic regions or climatic zones?

We hypothesised that butterfly populations will show local differences in their ability to cope with temperature changes, such that butterflies in warmer, drier climates, regardless of geographic region, will maintain more stable body temperatures than butterflies from cooler, wetter climates (Laird-Hopkins et al., 2023). We also hypothesised that butterfly populations will show local differences in their thermoregulatory mechanisms, such that butterflies from warmer, drier climates will use cooler microclimates to avoid heat stress more than butterflies from cooler, wetter climates (Ma et al., 2021; Pincebourde & Suppo, 2016). In contrast, butterflies from cooler, wetter climates will use warmer microclimates to gain heat more than butterflies from warmer, drier climates (Bonebrake et al., 2014; Kleckova & Klecka, 2016). Additionally, we expected butterflies from cooler, wetter climates to heat up more through postural means than butterflies from warmer, drier climates (Laird-Hopkins et al., 2023) and conversely, butterflies from warmer, drier climates to cool down more through postural means than butterflies from cooler, wetter climates (Bonebrake et al., 2014; Tsai et al., 2020).

3.3 Methods

3.3.1 Study sites

Central European butterflies were measured at 20 sites in the Czech Republic and Austria from May to August 2021 (Laird-Hopkins et al., 2023). Southern European butterflies were measured at five sites in northern Spain from March to October 2019–2020 (Toro-Delgado et al., In review). North-western European butterflies were measured at eight sites in the United Kingdom from April to September 2009 and 2018 (Bladon et al., 2020), with additional unpublished data collected at five of these sites and seven additional sites from 2019–2022.

Study sites included calcareous grasslands, secondary forest and alpine meadows (Supplementary Table 3.1).

3.3.2 Butterfly body temperature measurements

Sites were searched randomly on foot, and butterflies were caught with a net and held securely without touching them. Data were collected following Bladon *et al.* (2020), as follows. Within one minute of capture, and whilst still in the net, the temperature of the butterfly's thorax (body temperature, T_b) was taken using a 0.5 mm type K thermocouple and hand-held indicator (Tecpel Thermometer 305B, TC Direct, Uxbridge, United Kingdom). Air temperature (T_a) at waist height in the shade was recorded with the same thermocouple, at the location where the butterfly was caught. If the butterfly was perched before collection, the air temperature of the microclimate (microclimate temperature, T_m) 1 cm above the perch was recorded. The individual was identified to species.

3.3.3 Climatic zones

The temperature and precipitation of each study site was categorised according to bioclimatic region. We imported a high-resolution global environmental stratification map from Metzger *et al.* (2013) into QGIS 3.28.0 (QGIS Development Team, 2022) and extracted the bioclimatic classification of every field site based on its geographic coordinates (Fig. 3.1, Supplementary Table 3.1) (Metzger *et al.*, 2013). Our study sites fell within three temperature classifications (cold, cold temperate and warm temperate) and five precipitation classifications (xeric, dry, mesic, moist and wet). For analysis, xeric and dry sites were grouped together as dry sites, and moist and wet sites were grouped together as wet sites. This left three precipitation categories: dry, mesic and wet. In Central Europe there were two temperature zones (nine cold and 11 cold temperate sites) and three precipitation zones (eight dry, 10 mesic and two wet sites), in North-western Europe there were two temperature zones (three cold and 12 cold temperate sites) and one precipitation zone (15 wet sites), and in Southern Europe there were two temperature zones (one cold and four warm temperate sites) and two precipitation zones (three dry and two mesic sites). It is important to note that there is a degree of co-variation between climate zone and region, as can be seen from Fig. 3.1 and this was

considered when interpreting the results. Additionally, butterfly surveys took place across a range of different habitats, which whilst it strengthens our results as we cover multiple habitats, could have also influenced our results when comparing across geographic regions and climatic zones. This was also taken into consideration when interpreting the results.

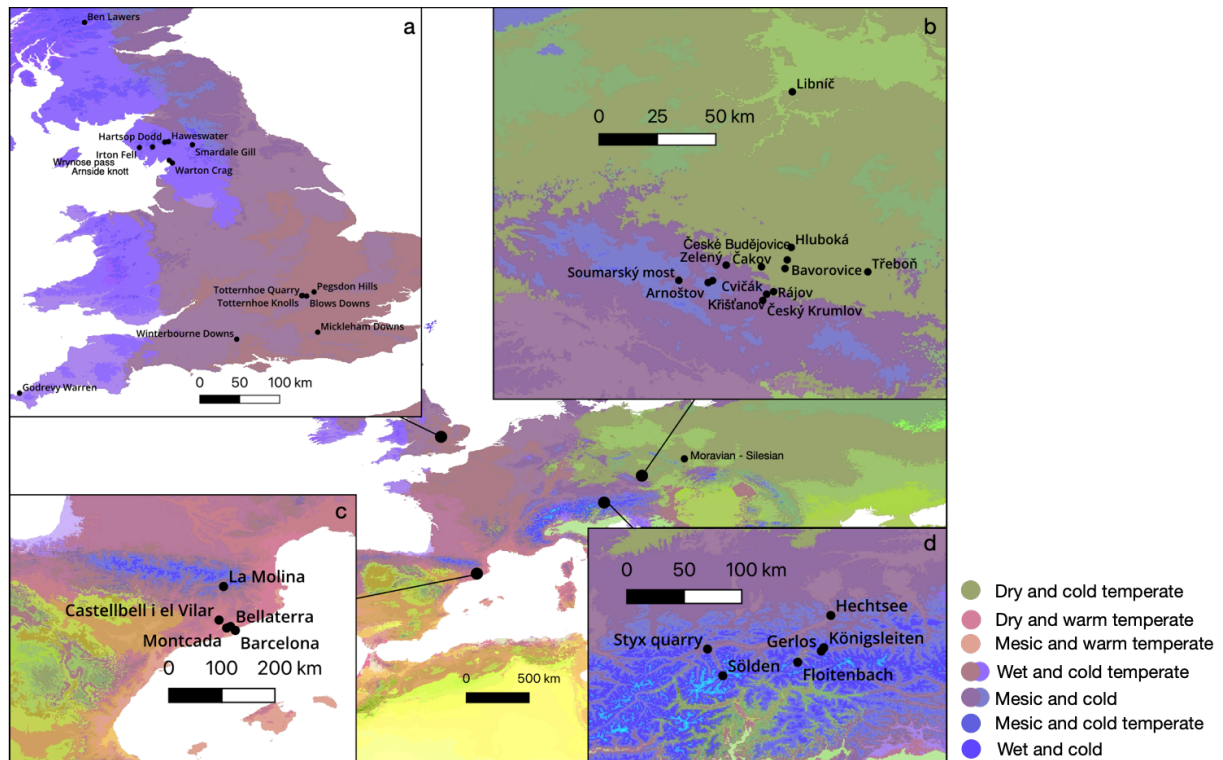


FIGURE 3.1. Field sites in A) the United Kingdom, B) the Czech Republic, C) Spain and D) Austria, overlaid on a map of European bioclimatic regions from Metzger *et al.* 2013.

3.3.4 Statistical analysis

All statistical analyses were undertaken in R version 4.1.2 (R Core Team, 2022). Graphics were produced using the package *ggplot2* (Wickham, 2016). To ensure normality and homogeneity in the residuals, graphical model diagnostics were checked for each model.

Calculation of buffering ability

To estimate the buffering ability of each species, simple linear regressions were fitted with T_b as the response variable and T_a as the predictor variable. Following Bladon *et al.* (2020), the slope of the relationship was extracted and subtracted from one to provide an estimate of buffering ability. A steep slope (low buffering ability) implies that the species' T_b varies greatly

over a small range of T_a , thus indicating a limited ability to maintain a stable body temperature. A shallow slope (high buffering ability) implies that the species' T_b remains relatively stable over a wide range of T_a , indicating a stronger ability to maintain a stable body temperature. Species with at least ten T_b measurements across a range of at least 5 °C of T_a were included in the analysis, to ensure our estimates of buffering ability were robust.

Does buffering ability differ between butterfly assemblages from different geographic regions and climatic zones?

To test whether assemblage-level buffering ability differs between butterfly communities in different geographic regions and climatic zones, three linear mixed-effects models (LMM) were fitted. In each model, individual butterfly T_b was the response variable, T_a and one of geographic region (Central, Southern and North-western Europe), precipitation (dry, mesic and wet) and temperature (cold, cold temperate and warm temperate) of the site where the butterfly was caught was fitted as a predictor variable. T_a at the time of capture was included in all the models, and the two-way interaction between T_a and the other term, were fitted as predictor variables in each model, such that a significant interaction indicates a difference in buffering ability between populations found in different locations. Species identity was included as a random effect. AIC model selection in the R package *cAIC4* (Säfken et al., 2021) was performed to determine if the full model was a better fit for the data than a model without the interaction term; if so, it suggests that butterflies from different locations differed in their thermal buffering ability. The best-fitting model with each of geographic region, precipitation and temperature as predictor variables were compared using AIC model selection to determine which of geographic region, precipitation and temperature best explained the data (Burnham & Anderson, 2004).

To test for a phylogenetic signal in the results, a European butterfly phylogeny (Wiemers et al., 2020) was pruned to the species present in our data, and used to fit three phylogenetic generalised linear mixed models (PGLMMs). The best-fitting model for each of geographic region, and precipitation and temperature zone was rerun using PGLMM in a Bayesian framework using the R package *phyr* (Li et al., 2020). Species and phylogeny were included as

random effects. Overall phylogenetic effect was calculated as the percentage of variance explained by phylogeny.

Does buffering ability differ between populations of individual species from different geographic regions and climatic zones?

To test whether individual species' buffering ability differs between populations living in different geographic regions or climatic zones, data were subset to species for which sufficient data (at least ten T_b measurements across a range of at least 5 °C of T_a) were available in two or more geographic regions (22 species), precipitation zones (23 species) or temperature zones (19 species). For each species, simple linear regressions were fitted with T_b as the response variable, and T_a and either geographic region, precipitation or temperature zone, and their interaction with T_a , as predictor variables. The R^2 was obtained using the R package *rr2* (Ives & Li, 2018) and used to compare models to determine which predictor best explained the data. For each model, an F-test was used to determine if the model with the interaction term was a better fit than the model without the term. A Benjamini-Hochberg correction was applied to the p-values to account for false positives, using the R package *FAS* (Ogle, 2016).

Calculation of microclimate selection and postural thermoregulation

For each individual butterfly, we calculated the extent to which its chosen T_m differed from the surrounding T_a , by subtracting T_a from T_m ("microclimate selection"), following Laird-Hopkins *et al.* (2023). A large difference between T_m and T_a suggests that the butterfly selected a specific thermal microclimate distinct from T_a . In addition, we calculated the extent to which each butterfly's T_b differed from the temperature of its chosen T_m , by subtracting T_m from T_b ("postural thermoregulation"), following Laird-Hopkins *et al.* (2023). A large difference between T_m and T_b suggests the butterfly was regulating its temperature independently of the microclimate, for example by orientating its wings relative to the sun to raise or lower its body temperature. Finally, we subtracted the microclimate selection value ($T_m - T_a$) from the postural thermoregulation value ($T_b - T_m$) for each butterfly, to calculate the index of thermal specialisation (ITS) (Bladon *et al.*, 2020; Laird-Hopkins *et al.*, 2023). Thus, when a butterfly uses postural thermoregulation, raising its body temperature beyond that of its microclimate,

it has a large ITS value. On the other hand, when a butterfly relies on selecting suitable microclimates for thermoregulation, it has a small ITS value. In order to include species as a random effect in the models, the data were subset to include only species with at least ten microclimate temperature measurements (T_m) across all regions.

Does the extent of thermoregulation via the use of microclimates or postural means, or the index of thermal specialisation, differ between butterfly assemblages from different geographic regions or climatic zones?

Three linear mixed-effects models (LMM) were fitted with each of the following variables: microclimate selection, postural thermoregulation and ITS separately, as response variables. Geographic region, precipitation zone and temperature zone were fitted separately as predictor variables for each response variable. T_a and the two-way interaction between T_a and the other variable were also fitted as predictor variables. Species was included as a random effect. AIC model selection was performed to determine if the full model was a better fit for the data than a model without the interaction term between region/zone and T_a ; if so, it suggests that butterflies from different regions/zones differed in their use of microclimate selection, postural thermoregulation or ITS across a range of T_a . AIC model selection was also performed to determine if the model including region/zone and T_a was a better fit than the model including only T_a ; if so, it suggests that butterflies from different regions/zones differed in their use of microclimate selection, postural thermoregulation and ITS, but independently of T_a . The best-fitting model with each of geographic region, precipitation and temperature as predictor variables were compared using AIC model selection to determine which of geographic region, precipitation and temperature best explained the data. The best-fitting models for each of geographic region, precipitation and temperature zone were rerun using PGLMMs (as described above) to test for a phylogenetic signal. Species and phylogeny were included as random effects. Overall phylogenetic effect was calculated as the percentage of variance explained by phylogeny.

3.4 Results

In total, 7,900 individuals of 59 species, across six families, were measured (Hesperiidae: 7 species; Lycaenidae: 10 species; Nymphalidae: 30 species; Papilionidae: 2 species; Pieridae: 9 species; Riodinidae: 1 species). Air temperature (T_a) ranged from 10.0 °C to 39.3 °C, with a mean of 22.2 °C. Body temperature (T_b) ranged from 14.1 °C to 45.0 °C, with a mean of 28.2 °C. Regional temperature ranges are presented in Supplementary Table 3.2.

3.4.1 Does buffering ability differ between butterfly assemblages from different geographic regions and climatic zones?

The butterfly assemblage from Southern Europe had the highest buffering ability (0.34 ± 0.02), followed by the assemblage from Central (0.29 ± 0.02) and North-Western Europe (0.24 ± 0.01) (Fig. 3.2a, Table 3.1a, Supplementary Table 3.2). Butterflies from mesic zones (0.32 ± 0.02) were better at buffering than those from wet (0.24 ± 0.01) or dry (0.24 ± 0.02) zones (Fig. 3.2b, Table 3.1a, Supplementary Table 3.2). Butterflies from warm temperate (0.33 ± 0.02) and cold (0.29 ± 0.03) zones were better at buffering than butterflies from cold temperate zones (0.23 ± 0.01) (Fig. 3.2c, Table 3.1a, Supplementary Table 3.2). The model with geographic region and the model with temperature zone as predictor variables fitted similarly to the data, and were a better fit than the model with precipitation zone as a predictor variable (Table 3.1a). Phylogeny accounted for 5.68%, 3.64% and 2.54% of the variation in the models with geographic region, precipitation and temperature zone, respectively (Supplementary Table 3.3a-c).

TABLE 3.1. AIC model comparisons, including Delta AICc and Akaike weight, for **(a)** buffering ability, **(b)** microclimate selection, **(c)** postural thermoregulation and **(d)** index of thermal specialisation. To aid comparison, models are present in the same order for each response variable, rather than ordered by AICc. The models with the most support (highest Akaike weight) for each response variable are highlighted in bold.

a. Buffering ability				
Fixed effects	Random effects	AICc	Delta AICc	Akaike weight
Air temperature + geographic region + air temperature:geographic region	Species	38310.20	0.50	0.497
Air temperature + geographic region	Species	38342.89	33.19	<0.001
Air temperature + precipitation zone + air temperature:precipitation zone	Species	38378.70	69.00	<0.001
Air temperature + precipitation zone	Species	38384.08	74.38	<0.001
Air temperature + temperature zone + air temperature:temperature zone	Species	38309.7	0.00	0.503
Air temperature + temperature zone	Species	38366.42	56.72	<0.001
b. Microclimate selection				
Fixed effects	Random effects	AICc	Delta AICc	Akaike weight
Air temperature + geographic region + air temperature:geographic region	Species	7509.37	4.11	<0.001
Air temperature + geographic region	Species	7505.26	0.00	0.504
Air temperature + precipitation zone + air temperature:precipitation zone	Species	7505.39	0.13	0.496
Air temperature + precipitation zone	Species	7512.77	7.51	<0.001
Air temperature + temperature zone + air temperature:temperature zone	Species	7520.55	15.29	<0.001
Air temperature + temperature zone	Species	7518.34	13.08	<0.001
Air temperature	Species	7526.07	20.81	<0.001
c. Postural thermoregulation				
Fixed effects	Random effects	AICc	Delta AICc	Akaike weight
Air temperature + geographic region + air temperature:geographic region	Species	8843.93	8.10	0.223
Air temperature + geographic region	Species	8860.17	24.34	<0.001
Air temperature + precipitation zone + air temperature:precipitation zone	Species	8850.95	15.12	0.095
Air temperature + precipitation zone	Species	8860.32	24.49	<0.001
Air temperature + temperature zone + air temperature:temperature zone	Species	8835.83	0.00	0.682
Air temperature + temperature zone	Species	8861.41	25.58	<0.001
Air temperature	Species	8858.84	23.01	<0.001
d. index of thermal specialisation				
Fixed effects	Random effects	AICc	Delta AICc	Akaike weight
Air temperature + geographic region + air temperature:geographic region	Species	10063.40	4.42	0.0952
Air temperature + geographic region	Species	10067.57	8.59	<0.001
Air temperature + precipitation zone + air temperature:precipitation zone	Species	10058.98	0.00	0.5681
Air temperature + precipitation zone	Species	10069.50	10.52	<0.001
Air temperature + temperature zone + air temperature:temperature zone	Species	10062.47	3.49	0.3367
Air temperature + temperature zone	Species	10070.53	11.55	<0.001
Air temperature	Species	10067.57	8.59	<0.001

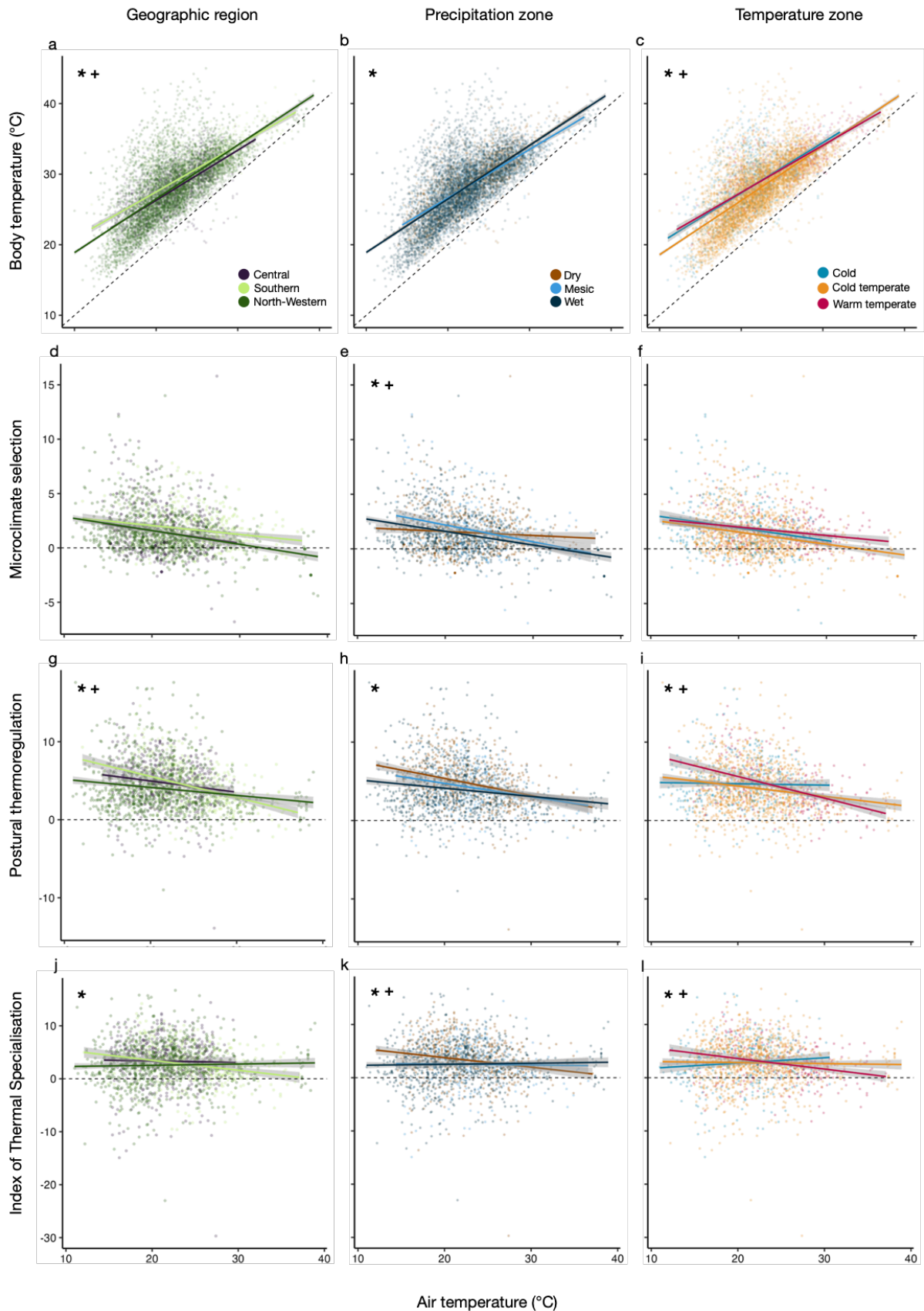


FIGURE 3.2. Individual butterfly (a-c) body temperatures (°C), (d-f) microclimate selection (the difference between microclimate temperature and air temperature), (g-i) postural thermoregulation (the difference between body temperature and microclimate temperature) and (j-l) index of thermal specialisation (postural thermoregulation – microclimate selection) at different air temperatures (°C)

from three geographic regions (Central, Southern and North-western Europe) (**a, d, g, j**), three climatic zones of precipitation (dry, mesic, wet) (**b, e, h, k**) and three climatic zones of temperature (cold, cold temperate and warm temperate) (**c, f, i, l**). Solid lines represent the modelled relationship. The grey bands show the 95% confidence intervals. An asterisk indicates where AIC model selection determined that the model including the two-way interaction term between air temperature (T_a) and region/zone was a better fit to the data than the model without the interaction term. A plus sign indicates the models with the most support (highest Akaike weight) for each response variable. The black dashed line represents a 1:1 relationship between body and air temperature (**a-c**) and zero (**d-l**), and are provided to facilitate comparison between plots.

3.4.2 Does buffering ability differ between populations of individual species from different geographic regions and climatic zones?

The model with geographic region and its interaction with T_a had the highest R^2 for six of 22 (27 %) species for which there was sufficient data to fit a model based on geographic region. One species (*Vanessa cardui*) showed intraspecific variation in buffering ability between geographic regions: butterflies from population in Southern Europe had lower buffering ability than butterflies from populations of the same species from North-western Europe (Fig. 3.3a, Supplementary Table 3.4-3.5). The model with precipitation zone and its interaction with T_a had the highest R^2 for four of 23 analysed species (17 %). Two species (*Lycaena phlaeas* and *V. cardui*) showed intraspecific variation in buffering ability between precipitation zones, with populations from mesic zones being better at buffering their T_b against changes in T_a than populations from wet or dry zones (Fig. 3.3b-c, Supplementary Table 3.4). The model with temperature zone and its interaction with T_a had the highest R^2 for four of 19 analysed species (21 %). Four species showed intraspecific variation in buffering ability between temperature zones. In two species (*L. phlaeas* and *V. cardui*), populations from cold zones had the highest buffering ability, while *Coenonympha pamphilus* populations from warm temperate zones and *Erebia epiphron* populations from cold temperate zones had the highest buffering ability, respectively (Fig. 3.3d-g, Supplementary Table 3.4).

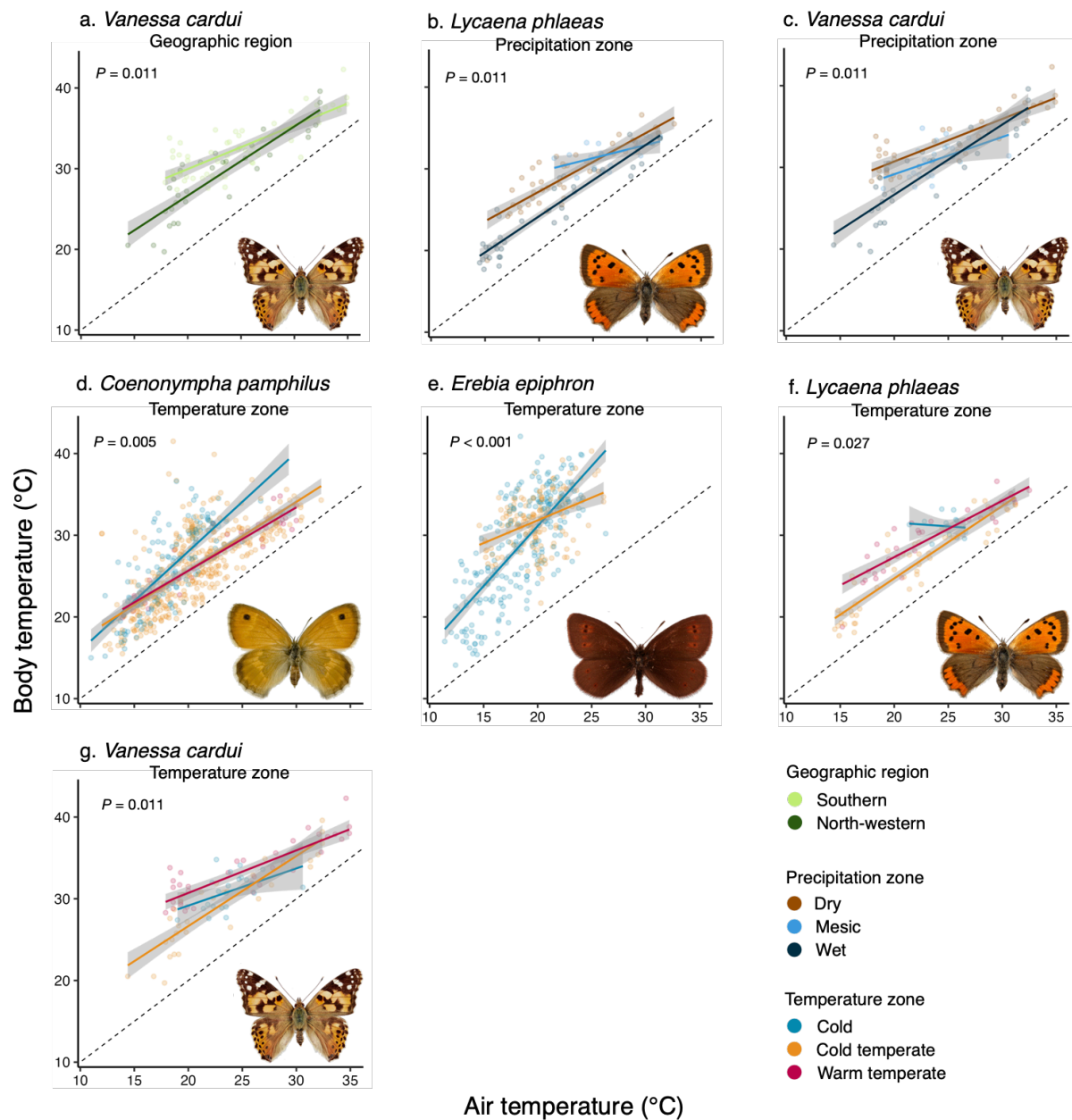


FIGURE 3.3. Individual butterfly body temperatures (°C) at different air temperatures (°C) from three geographic regions (Central, Southern and North-western Europe) **(a)**, three climatic zones of precipitation (dry, mesic, wet) **(b-c)** and three climatic zones of temperature (cold, cold temperate and warm temperate) **(d-g)** of *Coenonympha pamphilus* (temperature zone), *Erebia epiphron* (temperature zone), *Lycaena phlaeas* (precipitation and temperature zone) and *Vanessa cardui* (geographic region and precipitation and temperature zone). Solid lines represent the modelled relationship between body temperature and air temperature. The grey bands show the 95% confidence intervals. The black dashed line represents a 1:1 relationship between body and air temperature. The p-value from the likelihood ratio test comparing models with and without the interaction between air temperature and region/zone is shown. Only species with significant differences in buffering ability between geographic regions or climate zones are shown; for full results see Supplementary Table 3.4. See Supplementary Table 3.5 for inset photo credits. Photos are not to scale.

3.4.4 Does the extent of thermoregulation via the use of microclimates or postural means, or the index of thermal specialisation, differ between butterfly assemblages from different geographic regions or climatic zones?

A total of 42 species from six families (Hesperiidae: 6 species; Lycaenidae: 8 species; Nymphalidae: 20 species; Papilionidae: 1 species; Pieridae: 6 species; Riodinidae: 1 species; $n = 1,801$ individual butterflies) had at least ten microclimate measurements across all regions.

Microclimate selection

Butterflies from Southern European regions were found in warmer microclimates than butterflies from North-western and Central European regions (Fig. 3.2d, Table 3.1b, Supplementary Table 3.2). Butterflies from wet and mesic zones used microclimates at low temperatures, but not at high temperatures, whereas butterflies from dry zones maintained a more consistent use of microclimates across air temperatures (Fig. 3.2e, Table 3.1b, Supplementary Table 3.2). Independent of T_a , butterflies from cold zones were found in the warmest microclimates followed by butterflies from warm temperate and finally cold temperate zones (Fig. 3.2f, Table 3.1b, Supplementary Table 3.2). The model with geographic region and the model with precipitation zone as predictor variables fitted similarly to the data and were a better fit than the model with temperature zone as a predictor variable (Table 3.1b). Phylogeny accounted for <0.001% of the variation in the models with geographic region, precipitation and temperature (Supplementary Table 3.6a-c).

Postural thermoregulation

Butterflies from Southern Europe used postural thermoregulation to elevate their body temperature the most at low temperatures, and the least at high temperatures, while butterflies from the other two regions were more consistent in their use of postural thermoregulation over the range of air temperatures (Fig. 3.2g, Table 3.1c, Supplementary Table 3.2). Butterflies from dry zones varied the most over the range of air temperatures, with butterflies from wet zones being the most consistent, and butterflies from mesic zones being intermediate (Fig. 3.2h, Table 3.1c, Supplementary Table 3.2). Butterflies from warm

temperate zones showed the greatest variation in their use of postural thermoregulation across the range of air temperatures, whereas butterflies from cold zones showed the least variation and butterflies from cold temperate zones were intermediate (Fig. 3.2i, Table 3.1c, Supplementary Table 3.2). The model with temperature zone as a predictor variable fitted the data best, followed by the model with geographic region and the model with precipitation zone (Table 3.1c). Phylogeny accounted for 0.009%, 0.004% and 0.007% of the variation in the models with geographic region, precipitation and temperature, respectively (Supplementary Table 3.7a-c).

Index of thermal specialisation

The ITS for Southern European butterflies increased the most at low air temperatures, indicating they use postural thermoregulation more, and decreased the most at high air temperatures, indicating they use microclimates for thermoregulation more, while the ITS for butterflies from the other two regions was more consistent (Fig. 3.2j, Table 3.1d, Supplementary Table 3.2). Similarly, the ITS for butterflies from dry zones increased the most at low air temperatures and decreased the most at high temperatures, whereas the ITS for butterflies from the other precipitation zones was more consistent (Fig. 3.2k, Table 3.1d, Supplementary Table 3.2). The ITS for butterflies from cold zones increased with air temperature, whereas the opposite trend was found for butterflies from warm temperate regions and the ITS for butterflies from cold temperate regions was intermediate (Fig. 3.2l, Table 3.1d, Supplementary Table 3.2). The model with precipitation zone as a predictor variable fitted the data marginally better than the models with geographic region and temperature zone (Table 3.1d). Phylogeny accounted for 0.015%, 0.009% and 0.007% of the variation in the models with geographic region, precipitation and temperature, respectively (Supplementary Table 3.8a-c).

3.5 Discussion

At the assemblage level, the ability of butterflies to buffer body temperature against changes in air temperature differed between geographic regions and sites in different climatic zones. Temperature zone and geographic region best explained the variation in the data. This result

is expected, as the local temperatures a butterfly experiences are a major determinant of its thermoregulatory ability and tolerance (Dongmo et al., 2021; Fischer & Karl, 2010; Montejó-Kovacevich et al., 2020). Butterflies from warm temperate zones had the highest buffering ability, followed by butterflies from cold zones and finally butterflies from cold temperate zones. This was driven by butterflies from warm temperate zones being better able to elevate their body temperature at low temperatures, likely in an effort to increase activity time. Butterflies from Southern Europe had the highest buffering ability, followed by butterflies from Central and North-western Europe. This is probably the result of the higher solar radiation found at lower latitudes, which may provide butterflies with a greater opportunity to elevate their body temperature at low air temperatures through basking (postural thermoregulation) or through the higher availability of warm microclimates (Besharat et al., 2013; Bonebrake et al., 2014). Butterflies from mesic zones were better at buffering than those from dry and wet zones. However, the wet sites we sampled were all located in cold and cold temperate zones, with none located in warm temperate zones, thus the lower buffering ability of butterflies from wet sites may be due to sampling bias of the temperate zones. Alternatively, it may be that in wet zones the cloudier climate results in lower solar radiation, meaning that butterflies are less able to elevate their body temperature at low air temperatures. The difference in buffering ability between dry and mesic zones might be explained by the higher humidity in mesic zones reducing the risk of desiccation and hence being beneficial for thermoregulation (Chown et al., 2011). It may also be due to differences in vegetation types between these zones. Due to the greater complexity of vegetation in mesic zones, there is likely to be a greater heterogeneity of microclimates than is found in dry zones, and by utilising these microclimates butterflies can more effectively thermoregulate (Kleckova & Klecka, 2016; Ma et al., 2021). Indeed, we found that butterflies from mesic zones used warm microclimates for thermoregulation more than butterflies from dry zones. Phylogeny did not account for much of the variance in the data, suggesting more closely related butterflies are not more similar in their thermal capabilities. This is unsurprising considering the amount of intraspecific variation in buffering ability observed.

We found intraspecific differences in buffering ability in four species (*C. pamphilus*, *E. epiphron*, *L. phlaeas*, *V. cardui*). This result is supported by other studies, which show habitat-specific differences in thermoregulatory ability between populations (Dongmo et al., 2021;

Fischer & Karl, 2010; Kleckova & Klecka, 2016; Melero et al., 2022; Montejo-Kovacevich et al., 2020). Melero *et al.* (2022) found that any differences in butterfly population responses to climate are the result of local adaptation. However, we suggest that this could also be the consequence of acclimation or plasticity. Melero *et al.* (2022) found that *V. cardui* is locally adapted and that this adaptation was driven by precipitation, however, considering our results and that *V. cardui* is a migratory butterfly, we suggest that this is instead acclimation and that this species exhibits a high degree of plasticity in its thermoregulatory abilities, meaning it can reside across a variety of climates (Masters et al., 1988). Melero *et al.* (2022) show that *L. phlaeas* is not locally adapted, driven by temperature, whereas we found differences in *L. phlaeas*' buffering ability between populations from different climatic zones, which were likely driven by precipitation patterns. This discrepancy may be due to differences in the approach of our studies, for example, Melero *et al.* (2022) has focused on population trends, whereas our data is sampled at one time point, and requires further investigation. Considering only the 16 species which were included in both this study and Melero *et al.* (2022), we found little evidence supporting their results, which showed local adaptation or acclimation to climate in other European butterfly species where we did not find an effect. The two studies, ours and Melero *et al.* (2022), differ in their approach in a number of ways, which may explain the differences in our results. While we consider behavioural mechanisms as well as microclimate temperatures, Melero *et al.* (2022) focused on overall population trends and do not consider small scale changes in climates. Considering this, we highlight the importance of including behavioural and microclimate data, as well as species population data, at both the community and regional level when assessing climate sensitivity.

3.5.1 Microclimate selection, postural thermoregulation and index of thermal specialisation

Precipitation zone and geographic region explained the microclimate selection data better than temperature zone. We found that temperature zone explained postural thermoregulation data better than geographic region or precipitation zone. We found that precipitation zone explained the index of thermal specialisation (ITS) data marginally better than temperature zone and geographic region.

Butterflies from Southern Europe were found in warmer microclimates than those from other geographical regions which may be explained by the warmer climate and greater solar radiation of this region creating warmer microclimates. Butterflies from Southern Europe also used postural thermoregulation to raise their body temperature the most at low temperatures and the least at high temperatures, while butterflies from the other regions were more consistent in their use of postural thermoregulation. The ITS showed a similar trend and was likely driven by the pattern observed in postural thermoregulation. This may be due to the higher solar radiation of Southern Europe that allows butterflies to effectively gain heat through postural means when air temperatures are low (Bonebrake et al., 2014; Shanks et al., 2015).

Butterflies from mesic and wet zones were found in the warmest microclimates at low air temperatures and in the coolest microclimates at high air temperatures. On the other hand, butterflies from dry zones were more consistent in microclimate use as air temperature changed. Mesic and wet zones may have more vegetation and greater habitat heterogeneity, offering a greater availability of microclimates for thermoregulation, which butterflies use to warm up at cool air temperatures, and avoid heat stress at higher air temperatures (Kleckova & Klecka, 2016; Ma et al., 2021). Contrary to our hypothesis, at low air temperatures butterflies from dry zones used postural thermoregulation more than those from mesic or wet zones. This may be the consequence of lower cloud cover in dry zones resulting in higher solar radiation and hence greater opportunity for postural thermoregulation (Besharat et al., 2013; Bonebrake et al., 2014). Additionally, the lack of suitable microclimates in these zones may make thermoregulation via microclimate selection difficult and so these butterflies use postural means to gain heat instead. Indeed, the ITS showed that butterflies from dry zones relied more on thermoregulating through postural means than via the selection of microclimates at low air temperatures than butterflies from other precipitation zones. Across all zones, the use of postural thermoregulation decreased at high air temperatures, suggesting that butterflies are using postural thermoregulation to gain heat when air temperatures are low, not to lower their temperature at high air temperatures.

Butterflies from cold zones were found in warmer microclimates than those from other temperature zones. In cold zones, warm microclimates may be particularly beneficial to

butterflies, as it allows them to gain heat when air temperatures are low (Kleckova & Klecka, 2016). Butterflies from cold zones did not vary their use of postural thermoregulation across the range of air temperature, while the use of postural thermoregulation by butterflies from warm temperate zones decreased with increasing air temperatures. Butterflies from cold temperate zones were intermediate between these strategies. This may reflect the need for butterflies in colder regions to warm up at most of the air temperatures they experience to maximise activity, whereas in warm temperate locations they must balance warming up at low temperatures and avoiding heat stress at higher temperatures (Advani et al., 2019). Our results demonstrate that postural thermoregulation, rather than microclimate selection, is the main mechanism by which they achieve this. At high air temperatures butterflies from cold and cold temperate zones relied more on thermoregulation through postural means than via the selection of microclimates than butterflies from warm temperate zones, perhaps because the temperature a butterfly needs to achieve to stay active exceeds that of the available microclimates and so postural thermoregulation is favoured (Wenda et al., 2021).

3.5.2 Implications and conclusions

We show the importance of butterflies' thermoregulatory abilities at the community level. Our study suggests that butterflies have different thermoregulation behaviour depending on the region where they occur, which may be the result of local adaption or acclimatisation to different aspects of the climate they experience. Climate change will bring increasing mean temperatures, heatwave events and drought to Europe, which is already resulting in changes in the abundance and distribution of butterflies (Lee et al., 2021; Spinoni et al., 2018). Here we show that with increasing temperatures butterflies from dry and warm temperate zones will become more reliant on microclimates. Bladon *et al.* (2020) found that in the UK butterflies reliant on microclimates at high temperatures are declining faster than those more reliant on postural thermoregulation. In Europe butterflies from arid environments are exhibiting the fastest declines in abundance compared to those from other habitats (Barton et al., 2019; Herrando et al., 2019). In combination this suggests that butterflies relying on microclimates for cooling at high air temperatures are most at risk from increasing temperatures with climate change, explaining why butterfly populations in arid regions are declining. It is unclear to what extent acclimation and local adaptation may be able to help

buffer some of the changes in temperature as a result of climate change, but it may be insufficient to prevent significant changes in butterfly species distributions and local extinction (Garnas, 2018; Gunderson & Stillman, 2015; Seebacher et al., 2015). Either way it is likely that not all species will be impacted equally by climate change, and will depend on whether they are locally adapted, and the behavioural or morphological mechanism underpinning their adaptation (Melero et al., 2022). As vital components of many ecosystems, changes in the population dynamics of butterflies may have knock-on effects at the ecosystem level (Bonebrake et al., 2010). Our study highlights the importance of microclimates for thermoregulation, indicating that changes in land use which alter the availability of different microclimates may impact species' capacity to cope with climate change. Additionally, if local climatic conditions are driving butterfly thermoregulatory patterns, then we can begin to predict which habitats may be most impacted by climate change and target them for management, facilitating species' ability to thermoregulate.

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3.7 Supplementary material

SUPPLEMENTARY TABLE 3.1. *Study sites in Central, Southern and North-Western Europe, including coordinates, elevation (metres above sea level), geographic region, precipitation and temperature zone, country and land type.*

Site	Coordinates		Elevation	Geographic region	Precipitation zone	Temperature zone	Country	Land type
	N	E						
Arnoštov	48.894660	13.994615	840	Central Europe	Mesic	Cold	Czech Republic	Grassland meadows
Arnside Knott	54.188483	-2.832434	87	North-Western Europe	Wet	Cold temperate	United Kingdom	Calcareous grassland
Barcelona	41.425174	2.199996	10	Southern Europe	Dry	Warm temperate	Spain	Urban ruderal meadow
Bavorovice	49.023401	14.443310	375	Central Europe	Dry	Cold temperate	Czech Republic	Grassland meadows
Bellaterra	41.506389	2.100000	155	Southern Europe	Dry	Warm temperate	Spain	Grassland meadows and Mediterranean forest
Ben Lawers	56.532870	-4.273260	614	North-Western Europe	Wet	Cold	United Kingdom	Montane grassland
Blows Downs	51.883210	-0.495800	202	North-Western Europe	Wet	Cold temperate	United Kingdom	Calcareous grassland and scrub
Čakov	48.984240	14.296788	426	Central Europe	Dry	Cold temperate	Czech Republic	Grassland meadows
Castellbell i el Vilar	41.640483	1.861652	170	Southern Europe	Mesic	Warm temperate	Spain	Encroaching scrubs and sparse Mediterranean forest
České Budějovice	48.974414	14.430564	442	Central Europe	Dry	Cold temperate	Czech Republic	Grassland meadows and secondary forest edge
Český Krumlov	48.793907	14.304518	503	Central Europe	Dry	Cold temperate	Czech Republic	Grassland meadows, roadside verges, secondary forest
Cvičák	48.829148	14.326293	591	Central Europe	Mesic	Cold	Czech Republic	Grassland meadows, encroaching scrub and marshland
Floitenbach	47.099153	11.810562	996	Central Europe	Mesic	Cold	Austria	Exposed ground, alpine grassland meadows, encroaching scrub and secondary forest
Gerlos	47.218297	12.062329	1346	Central Europe	Mesic	Cold	Austria	Exposed ground, alpine grassland meadows and forest edge

Godrevy Warren	50.234130	-5.379180	60	North-Western Europe	Wet	Cold temperate	United Kingdom	Grassland
Hartsop Dodd	54.498100	-2.909400	604	North-Western Europe	Wet	Cold	United Kingdom	Montane grassland
Haweswater	54.507560	-2.845980	652	North-Western Europe	Wet	Cold	United Kingdom	Montane grassland
Hechtsee	47.599623	12.164717	670	Central Europe	Wet	Cold temperate	Austria	Forest edge
Hluboká	49.094034	14.465941	405	Central Europe	Dry	Cold temperate	Czech Republic	Grassland meadows and secondary forest
Irton Fell	54.406720	-3.340000	236	North-Western Europe	Wet	Cold temperate	United Kingdom	Lowland grassland
Königsleiten	47.253974	12.089779	2050	Central Europe	Mesic	Cold	Austria	High alpine grassland meadow
Křišťanov	48.906300	14.019557	940	Central Europe	Mesic	Cold	Czech Republic	Grassland meadows
La Molina	42.335224	1.954362	1532	Southern Europe	Mesic	Cold	Spain	Grassland meadows alternated with montane conifer forest (mainly <i>Pinus sylvestris</i>)
Libníč	49.975953	14.472213	333	Central Europe	Dry	Cold temperate	Czech Republic	Grassland meadows
Mickleham Downs	51.269223	-0.307703	150	North-Western Europe	Wet	Cold temperate	United Kingdom	Calcareous grassland and mixed woodland
Montcada	41.476071	2.020043	74	Southern Europe	Dry	Warm temperate	Spain	Grassland meadows, encroaching scrub and mediterranean Holm oak forest
Moravian-Silesian	50.056424	17.228311	1444	Central Europe	Mesic	Cold temperate	Czech Republic	Grassland meadows
Pegsdon Hills	51.953540	-0.370200	100	North-Western Europe	Wet	Cold temperate	United Kingdom	Calcareous grassland and scrub
Rájov	48.843466	14.365909	459	Central Europe	Dry	Cold temperate	Czech Republic	Grassland meadows
Smardale Gill	54.454435	-2.438830	0	North-Western Europe	Wet	Cold temperate	United Kingdom	Calcareous grassland
Sölden	46.954529	11.008711	1511	Central Europe	Mesic	Cold	Austria	Exposed ground, alpine grassland meadows, encroaching scrub and secondary forest

Soumarský most	48.907138	13.827730	754	Central Europe	Mesic	Cold	Czech Republic	Grassland meadows and secondary forest
Styx quarry	47.239532	10.844794	733	Central Europe	Wet	Cold temperate	Austria	Exposed ground, alpine grassland meadows, forest edge and secondary forest
Totternhoe Knolls	51.889890	-0.580390	134	North-Western Europe	Wet	Cold temperate	United Kingdom	Calcareous grassland and scrub
Totternhoe Quarry	51.891990	-0.568360	125	North-Western Europe	Wet	Cold temperate	United Kingdom	Calcareous grassland and scrub
Třeboň	48.955848	14.900563	446	Central Europe	Dry	Cold temperate	Czech Republic	Grassland meadows
Warton Crag	54.144385	-2.779431	53	North-Western Europe	Wet	Cold temperate	United Kingdom	Calcareous grassland
Winterbourne Downs	51.149630	-1.685000	114	North-Western Europe	Wet	Cold temperate	United Kingdom	Calcareous grassland
Wrynose Pass	54.416667	-3.116667	425	North-Western Europe	Wet	Cold temperate	United Kingdom	Montane grassland
Zelený	48.993960	14.096682	805	Central Europe	Mesic	Cold	Czech Republic	Grassland meadows

SUPPLEMENTARY TABLE 3.2. *Assemblage-level buffering ability estimate (calculated as the slope of the simple linear regression of body temperature against air temperature, subtracted from 1, following Bladon et al. 2020), microclimate selection, postural thermoregulation and index of thermal specialisation (ITS), and their corresponding standard errors, sample size (n) and minimum, maximum and mean air temperature (°C) by geographic region, and precipitation and temperature climatic zones.*

Region/Zone		Air temperature / °C			Buffering ability estimate			Microclimate selection			Postural thermoregulation			ITS		
		min	max	$\bar{x}$	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n
Geographic region	Southern Europe	12.1	37.1	24.7	0.34	0.02	820	1.68	0.08	448	4.29	0.14	448	2.61	0.18	448
	North-Western Europe	10.0	39.3	21.9	0.24	0.01	5196	1.40	0.07	417	4.76	0.11	417	2.59	0.13	417
	Central Europe	14.4	32.2	22.2	0.29	0.02	1884	1.43	0.11	936	4.29	0.14	936	3.33	0.22	936
Precipitation zone	Dry	12.1	37.1	22.8	0.24	0.02	1680	1.50	0.08	569	4.79	0.12	569	3.30	0.17	569
	Mesic	14.4	36.7	23.0	0.32	0.02	945	1.63	0.13	286	4.13	0.16	286	2.49	0.25	286
	Wet	10.0	39.3	21.9	0.24	0.01	5275	1.41	0.07	946	3.96	0.11	946	2.56	0.13	946
Temperature zone	Cold	11.0	32.1	21.4	0.29	0.03	1240	1.86	0.12	377	4.68	0.15	377	2.81	0.22	377
	Cold temperate	10.0	39.3	22.2	0.23	0.01	6151	1.31	0.06	1142	4.12	0.09	1142	2.81	0.13	1142
	Warm temperate	12.1	37.1	24.9	0.33	0.02	509	1.61	0.09	282	4.21	0.17	282	2.60	0.21	282

SUPPLEMENTARY TABLE 3.3. *Model output for phylogenetic generalised linear mixed models (PGLMMs) of the effect of a. geographic region, b. precipitation zone and c. temperature zone on butterfly buffering ability (calculated as the slope of the regression of thoracic temperature against air temperature, subtracted from 1, following Bladon et al 2020). Bold indicates significant effects; coefficients where the 95% Bayesian credible interval (CI) does not overlap zero.*

a. Geographic region				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	0.790	0.889	0.485	1.587
1 phylogeny	0.494	0.703	0.230	1.063
residual	7.416	2.723	7.187	7.655
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	11.063	10.203	11.922	
Tair	0.762	0.723	0.800	
Region Southern Europe	3.517	2.104	4.930	
Region North Western Europe	-0.528	-1.477	0.420	
Tair:Region Southern Europe	-0.102	-0.161	-0.043	
Tair:Region North Western Europe	0.045	0.003	0.087	
b. Precipitation zone				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	1.222	1.105	0.776	2.214
1 phylogeny	0.328	0.573	0.108	1.025
residual	7.478	2.735	7.179	7.725
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	11.791	10.980	12.603	
Tair	0.750	0.715	0.785	
Rainfall Mesic	-0.212	-1.656	1.231	
Rainfall Wet	-1.184	-2.089	-0.280	
Tair:Rainfall Mesic	0.004	-0.057	0.065	
Tair:Rainfall Wet	0.053	0.014	0.092	
c. Temperature zone				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	1.252	1.119	0.814	2.138
1 phylogeny	0.226	0.476	0.092	0.607
residual	7.415	2.723	7.186	7.654
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	8.161	7.140	9.181	
Tair	0.920	0.874	0.966	
Temperature Cold temperate	2.685	1.620	3.751	
Temperature Warm temperate	6.239	4.637	7.841	
Tair:Temperature Cold temperate	-0.139	-0.188	-0.091	
Tair:Temperature Warm temperate	-0.257	-0.324	-0.190	

SUPPLEMENTARY TABLE 3.4. *Species-specific buffering ability estimates (BAE; calculated as the slope of the simple linear regression of body temperature against air temperature, subtracted from 1, following Bladon et al. 2020) and standard error (SE) in each geographic region (Central, Southern and North-western Europe), precipitation zone (dry, mesic and wet) and temperature zone (cold, cold temperate and warm temperate), with the number of individuals (n) sampled in each location, the R^2 of the maximal model (R^2), and the results of the likelihood ratio test (test statistic (F), degrees of freedom (df) and likelihood p-value (p)) between models with and without an interaction between air temperature and geographic region/climatic zone. Statistically significant likelihood p values are marked in bold and indicate a significant difference in buffering ability between populations from the different geographic regions or precipitation or temperature zones. In such cases, the region/zone with the highest buffering ability estimate is marked in bold. The highest R^2 across models for each species is marked in bold, indicating which of geographic region, precipitation or temperature zone best explain the data. All p-values are Benjamini-Hochberg adjusted.*

Species	Family	Geographic region													Precipitation zone													Temperature zone																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
		Central Europe				North-Western Europe				Southern Europe				R2	F	df	p	Dry				Mesic				Wet				R2	F	df	p	Cold				Cold temperate				Warm temperate				R2	F	df	p																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																
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<i>Aglais io</i>	Nymphalidae	0.31	0.08	75	0.50	0.20	16	-	-	-	-	0.481	1.028	1	0.507	0.28	0.08	72	1.53	0.13	3	0.50	0.20	16	0.515	1.408	1	0.425	-0.35	0.13	3	0.33	0.07	88	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

SUPPLEMENTARY TABLE 3.5. *Photo credits for inset photos in Fig. 3.3.*

Species	Photo credit	License
<i>Coenonympha pamphilus</i>	Pekka Malinen	CC BY-SA 4.0
<i>Erebia epiphron</i>	M. Fatahi	All rights reserved - please ask for the terms of use if required
<i>Lycaena phlaeas</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0
<i>Vanessa cardui</i>	Malinen, Pekka, Luomus	CC BY-SA 4.0

SUPPLEMENTARY TABLE 3.6. *Model output for phylogenetic generalised linear mixed models (PGLMMs) of the effect of a. geographic region, b. precipitation zone and c. temperature zone on butterfly microclimate selection. Bold indicates significant effects; coefficients where the 95% Bayesian credible interval (CI) does not overlap zero.*

a. Geographic region				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	0.143	0.379	0.073	0.318
1 phylogeny	0.000	0.009	0.000	0.001
residual	3.714	1.927	3.478	3.971
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	3.4285	2.9239	3.9320	
Tair	-0.0917	-0.1130	-0.0703	
Region Southern Europe	0.4996	0.1853	0.8115	
Region North Western Europe	-0.1876	-0.4503	0.0723	
b. Precipitation zone				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	0.166	0.408	0.086	0.366
1 phylogeny	0.000	0.007	0.000	0.001
residual	3.706	1.925	3.473	3.969
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	2.411	1.543	3.280	
Tair	-0.040	-0.077	-0.003	
Rainfall Mesic	2.886	1.282	4.489	
Rainfall Wet	0.892	-0.170	1.952	
Tair:Rainfall Mesic	-0.114	-0.181	-0.047	
Tair:Rainfall Wet	-0.053	-0.099	-0.007	
c. Temperature zone				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	0.139	0.373	0.082	0.332
1 phylogeny	0.000	0.004	0.000	0.021
residual	3.747	1.936	3.505	4.003
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	3.495	2.973	4.013	
Tair	-0.087	-0.109	-0.066	
Temperature Cold temperate	-0.275	-0.534	-0.014	
Temperature Warm temperate	0.289	-0.057	0.635	

SUPPLEMENTARY TABLE 3.7. *Model output for phylogenetic generalised linear mixed models (PGLMMs) of the effect of a. geographic region, b. precipitation zone and c. temperature zone on butterfly postural thermoregulation. Bold indicates significant effects; coefficients where the 95% Bayesian credible interval (CI) does not overlap zero.*

a. Geographic region				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	0.754	0.869	0.361	1.981
1 phylogeny	0.100	0.316	0.024	0.632
residual	7.746	2.783	7.266	8.324
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	7.705	6.041	9.369	
Tair	-0.144	-0.220	-0.068	
Region Southern Europe	3.329	1.074	5.584	
Region North Western Europe	-0.964	-2.807	0.879	
Tair:Region Southern Europe	-0.122	-0.220	-0.025	
Tair:Region North Western Europe	0.043	-0.041	0.127	
b. Precipitation zone				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	0.846	0.920	0.411	2.240
1 phylogeny	0.050	0.224	0.010	0.558
residual	7.788	2.791	7.288	8.327
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	9.843	8.565	11.120	
Tair	-0.226	-0.280	-0.171	
Rainfall Mesic	-2.265	-4.627	0.097	
Rainfall Wet	-3.068	-4.617	-1.519	
Tair:Rainfall Mesic	0.090	-0.009	0.188	
Tair:Rainfall Wet	0.120	0.053	0.187	
c. Temperature zone				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	0.800	0.894	0.390	1.927
1 phylogeny	0.087	0.295	0.021	0.593
residual	7.719	2.778	7.229	8.257
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	4.495	2.792	6.198	
Tair	0.014	-0.064	0.091	
Temperature Cold temperate	3.118	1.262	4.975	
Temperature Warm temperate	6.496	4.112	8.880	
Tair:Temperature Cold temperate	-0.155	-0.240	-0.071	
Tair:Temperature Warm temperate	-0.279	-0.380	-0.179	

SUPPLEMENTARY TABLE 3.8. *Model output for phylogenetic generalised linear mixed models (PGLMMs) of the effect of a. geographic region, b. precipitation zone and c. temperature zone on butterfly index of thermal specialisation. Bold indicates significant effects; coefficients where the 95% Bayesian credible interval (CI) does not overlap zero.*

a. Geographic region				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	0.760	0.872	0.301	3.781
1 phylogeny	0.092	0.304	0.018	0.818
residual	15.295	3.911	14.015	16.399
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	4.257	1.917	6.596	
Tair	-0.052	-0.159	0.055	
Region Southern Europe	3.083	-0.088	6.255	
Region North Western Europe	-0.739	-3.329	1.851	
Tair:Region Southern Europe	-0.132	-0.269	0.005	
Tair:Region North Western Europe	0.042	-0.076	0.160	
b. Precipitation zone				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	0.858	0.926	0.350	4.539
1 phylogeny	0.058	0.240	0.011	0.666
residual	15.294	3.911	14.139	16.350
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	7.380	5.601	9.159	
Tair	-0.183	-0.259	-0.107	
Rainfallmesic	-5.172	-8.461	-1.882	
Rainfallwet	-3.867	-6.021	-1.712	
Tair:Rainfallmesic	0.203	0.066	0.340	
Tair:Rainfallwet	0.168	0.075	0.261	
c. Temperature zone				
Random effects				
	Variance	Std.Dev	lower.CI	upper.CI
1 species	0.812	0.901	0.369	2.405
1 phylogeny	0.041	0.203	0.007	0.707
residual	15.277	3.909	14.304	16.617
Fixed effects				
	Estimate	lower.CI	upper.CI	
(Intercept)	1.645	-0.706	3.998	
Tair	0.071	-0.036	0.178	
TempCold temperate	2.621	0.046	5.196	
TempWarm temperate	5.850	2.534	9.165	
Tair:TempCold temperate	-0.120	-0.237	-0.002	
Tair:TempWarm temperate	-0.260	-0.400	-0.120	

Chapter 4

Morphology and habitat preference, not phylogeny, drive thermoregulation in mimetic *Heliconius* butterflies

Laird-Hopkins, B. C., Sam, K., Kleckova, I., Paneso, O., McMillian, W. O., & Nadeau N. J. (*in prep.*). Morphology and habitat preference, not phylogeny, drive thermoregulation in mimetic *Heliconius* butterflies. *Journal of Animal Ecology*.

4.1 Abstract

It remains unclear how tropical ectotherms respond to the likely changes in temperature caused by global warming. To understand this, we studied thermoregulation in *Heliconius* butterflies from Panama and Ecuador. Firstly, we utilised the Müllerian mimicry exhibited in this genus to tease apart the role of morphology and relatedness in thermoregulation, hypothesising that morphology, not phylogeny, drives thermoregulatory ability. Secondly, we compared the thermoregulatory ability of closely related species from different habitats, hypothesising that butterflies from hot environments will cope better with increases in temperatures than butterflies from cold environments. To do this, we tested buffering ability (ability to regulate body temperature relative to surrounding air temperature) in the field and, take-off temperature, and heating rate of both live and dead butterflies in the laboratory. Morphological traits, including colour, size and iridescence, not phylogeny, drove thermoregulation in *Heliconius* butterflies. The morphological traits driving heating rate differed between dead and live butterflies showing that morphology as well as behaviour and/or physiology determine *Heliconius* thermoregulatory ability. Butterflies from colder habitats were better at buffering temperatures and took off at a lower temperature than those from hotter habitats indicating that these species are adapted or acclimatised to the specific thermal environment they inhabit. Our results demonstrate that butterflies have unique and specific thermoregulatory strategies as a result of their morphology, behaviour and physiology, as well as the thermal environment they inhabit. This has important consequences for predicting how butterflies will be impacted with climate and land-use change.

4.2 Introduction

Climate change, through rising temperatures and extreme temperature events, is causing shifts in species distributions and phenology, with knock-on effects being seen at the community and ecosystem level (Parmesan, 2006). Increasing temperatures will continue to have far reaching consequences for species as environmental temperature influences many physiological processes (Angilletta, 2009). In this respect, tropical species are thought to be particularly vulnerable to climate change as they live closer to their thermal maximum and experience a narrower range of temperatures than temperate species (Deutsch et al., 2008; Janzen, 1967; Sunday et al., 2019). Temperatures in the tropics are expected to increase by up to 5 °C by 2100, with an increasing frequency of heatwave events (Lee et al., 2021). The groups most vulnerable to climate change and the extent that they will adapt to counteract the negative implications of rising temperatures remains hard to predict, especially given the paucity of information about thermoregulatory mechanisms employed by most tropical organisms.

Being ectotherms, butterflies are particularly vulnerable to changing temperatures and understanding their thermoregulatory abilities will help us gain a greater understanding of ecosystem-wide consequences of climate change (Bonebrake et al., 2010; Harvey et al., 2020). Morphology, physiology, behaviour and habitat use collectively influence butterfly thermoregulation (Bladon et al., 2020; Kleckova & Klecka, 2016; Laird-Hopkins et al., 2023). Indeed, morphological traits including size, colour, and reflectance have all been demonstrated to affect the thermoregulatory ability of butterflies (Bladon et al., 2020; Shanks et al., 2015; Tang et al., 2022). Butterflies also thermoregulate by employing specific behaviours, such as adjusting the positioning of their wings in relation to the sun, or using favourable microclimates (Kemp & Krockenberger, 2002; Laird-Hopkins et al., 2023; Shanks et al., 2015). Active cooling may be especially useful in lowland tropical forests, where microclimate temperatures can be 2 °C cooler than ambient temperatures, which often approach a species thermal maximum during the middle of the day (Deutsch et al., 2008; Montejo-Kovacevich et al., 2020). Finally, there is mounting evidence demonstrating that butterflies using different habitats differ in their thermoregulatory abilities and are adapted or acclimatised to the specific environmental conditions they experience (Dongmo et al.,

2021; Kleckova & Klecka, 2016; Montejo-Kovacevich et al., 2020). For example, high altitude butterflies are often adapted to take-off at lower temperatures than low altitude butterflies, and may have a lower thermal tolerance than lowland species (Advani et al., 2019; Montejo-Kovacevich et al., 2020). More closely related species tend to have similar thermoregulatory abilities due to sharing multiple aspects of physiology, morphology and behaviour. For example, Pieridae are better at thermoregulating than other butterfly families, perhaps as a consequence of being large and light-coloured (Bladon et al., 2020).

Understanding how species will respond to changing temperatures requires knowledge of how morphological and behavioural traits drive thermoregulation and if these traits have a phylogenetic component. In this light, *Heliconius* butterflies provide an excellent study system. The group evolved rapidly and inhabit a wide range of habitats across a broad elevational gradient throughout the Neotropics (Jiggins, 2017). Wing pattern variation is rampant in the group and the genus characterized by extensive convergent and divergent evolution. The result is that distantly related, but co-occurring species, often exhibit the same colouration whereas closely related species, or regional populations of the same species, often have a very different wing pattern (Fig. 4.1). This repeated pattern of convergent and divergent evolution is best exemplified by morphological change in two of the major lineages within the genus, the *H. erato/sapho/sara* (ESS) and the *H. melpomene/cydno/silvaniform* (MCS) species groups. The two lineages have been evolving separately for the past 12 million years, diverging in many aspects of their ecology and reproductive biology (Jiggins, 2017). Nonetheless, within the last few million years the groups have converged on a similar complement of wing pattern types, including many independent cases of near-perfect wing pattern mimicry.

We took advantage of this evolutionary mosaic to untangle the relative contributions of morphology and relatedness in butterfly thermoregulation. Any effect of relatedness would be indirect via a phylogenetic signal in functional traits involved in thermoregulation. Specifically, we used a combination of field and laboratory experiments in butterflies occupying different tropical habitats to gain a greater understanding of a butterfly's thermal capabilities across their activity period (Fig. 4.2). We hypothesised that (1) morphology will be more important than phylogenetic relatedness in thermoregulatory abilities such that

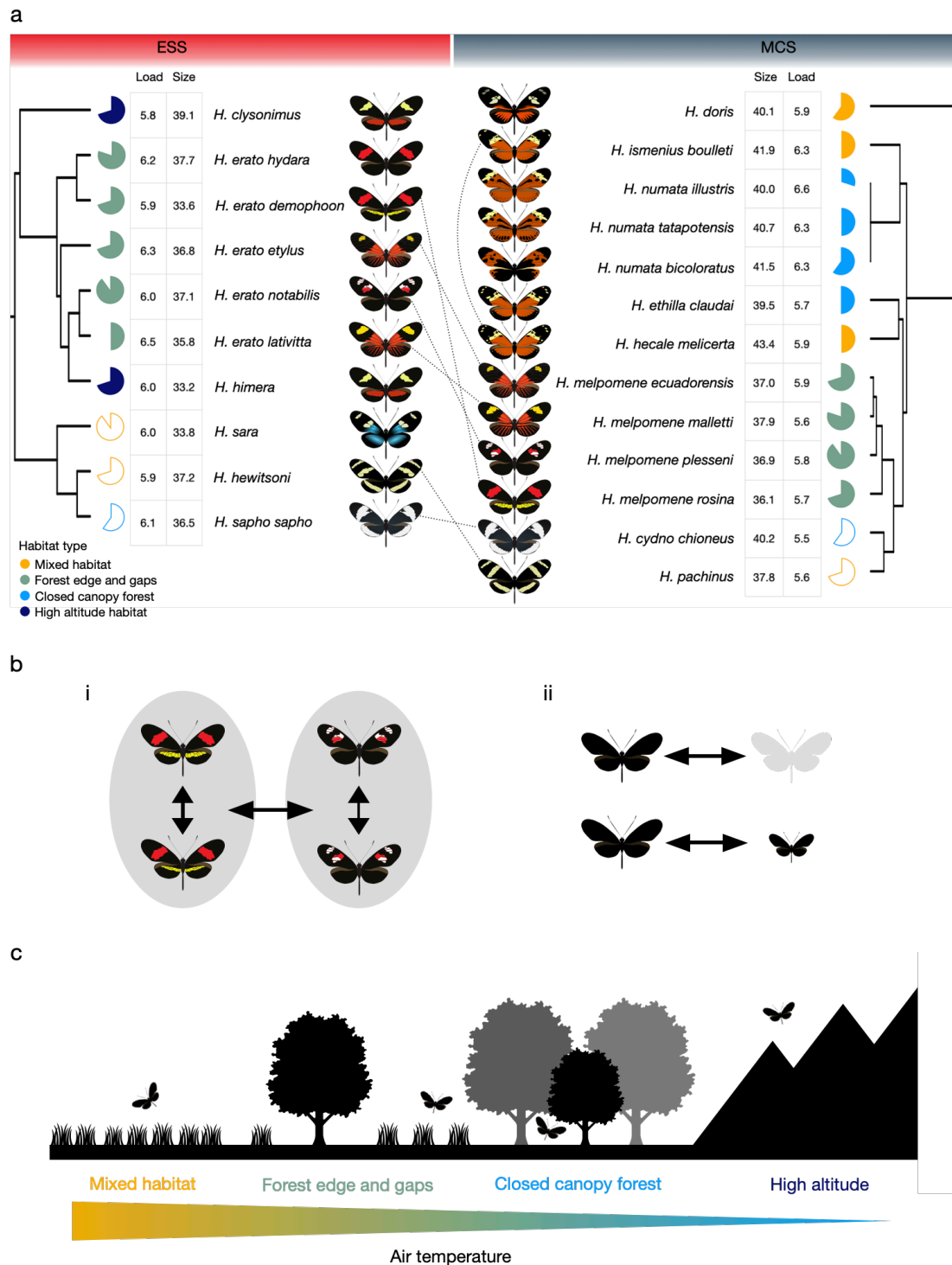


FIGURE 4.1. Firstly, we tested how morphology (including colour and size) (bi and bii) and relatedness determine thermoregulatory ability in wild and insectary reared *Heliconius* butterflies (a). We compared thermoregulatory ability of mimetic subspecies and grouped subspecies by their mimetic pair to compare thermoregulatory ability between pairs (bi). Secondly, we determined how *Heliconius* butterflies from different habitat types (mixed habitat, forest edge and gaps, closed canopy forest and high-altitude habitat) differed in their thermoregulatory ability (c). Mean wing load (mean thorax diameter:mean forewing length) and forewing size (mm) are given for the Erato/Sara/Sapho (ESS) and the Melpomene/Cydney/Sylvaniform (MCS) clade (a). Pie charts indicate percentage of black on the wings, a proxy for colour lightness, with colour indicating habitat type and unfilled pie charts indicating iridescence on the wings (a). Phylogeny was adapted from Cicconardi et al. (2023). Butterfly illustration credit: Steven Van Belleghem.

morphologically dissimilar, but closely related species will differ in their thermoregulatory ability, and that morphologically similar, but more distantly related species (mimetic pairs) will show similarities in thermoregulatory ability (Fig. 4.1 a-b). We also hypothesised that (2) species inhabiting hotter environments (forest edges and gaps) will have mechanisms to avoid overheating, and so will i) be better at buffering their body temperature against changes in air temperature, ii) be able take-off at lower temperatures and iii) heat up at a slower rate, than butterflies from cooler regions (closed canopy and high-altitude) (Fig. 4.1 c).

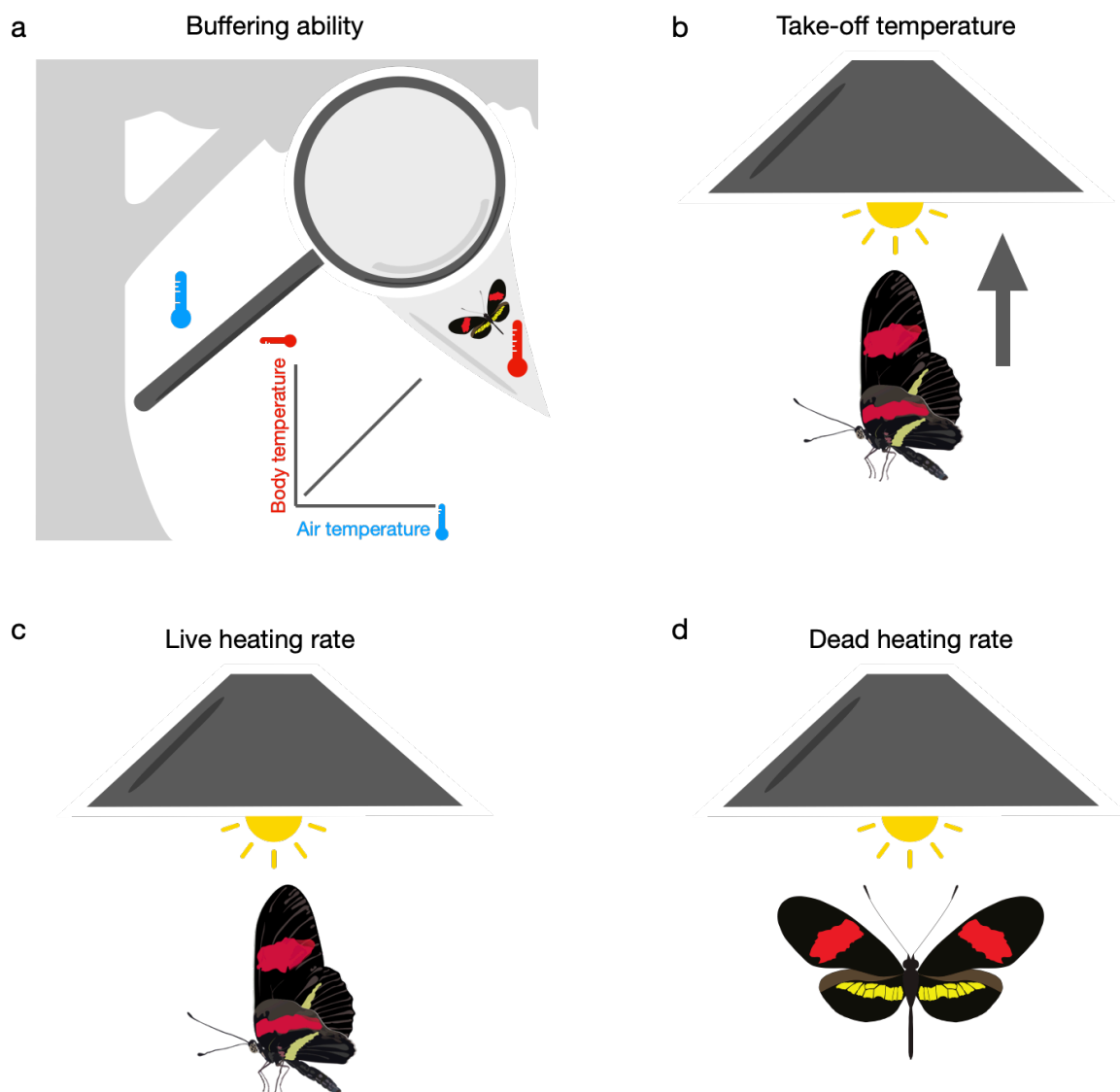


FIGURE 4.2. We studied ecologically relevant thermoregulatory traits to gain an understanding of how *Heliconius* butterflies respond to temperature. In the wild, we studied the thermal buffering ability, ability to regulate body temperature in response to changes in surrounding air temperature, of butterflies (a). In the laboratory, using wild caught and insectary reared specimens we determined the temperature at which butterflies start flying (b) and the rate at which butterflies heat up when alive (c) and dead (d). Butterfly illustration credit: Steven Van Belleghem.

4.3 Methods

4.3.1 Buffering ability measurements

Field work was undertaken between November 2021 - January 2022 and May - July 2022 in Panama across a range of habitat types (Supplementary Table 4.1). Field sites were randomly walked, and all observed butterflies were caught in nets. A temperature reading of the butterfly's thorax (T_b) was taken using a 0.25 mm type K thermocouple and hand-held indicator (TC Direct, Uxbridge, United Kingdom) within 10 seconds of capture. Care was taken to ensure that the butterfly was not touched directly to avoid artificially increasing its thorax temperature. Using the same thermocouple, a temperature of the air was recorded at waist height in the shade (T_a). Using a 1-5 scale, the butterfly's condition was recorded, 1 indicating a perfect butterfly and 5 indicating a badly damaged butterfly. The butterfly was identified to species or subspecies level and sexed. The forewing length (mm), forewing width (mm) and thorax diameter (mm) were measured using callipers on a subset of butterflies (minimum three individuals per species/subspecies per sex) (Hook et al., 2012). Mean forewing length, forewing width and thorax diameter was calculated per species/subspecies per sex. Mean forewing aspect ratio (mean forewing length:mean forewing width) and a proxy of mean wing loading (mean thorax diameter:mean forewing length) were calculated per species/subspecies per sex. For wing loading, forewing length was used as a proxy for wing area and thorax diameter was used as a proxy for fresh weight.

4.3.2 Take-off temperature and heating rate measurements

Laboratory work was undertaken between May – July 2022 in Gamboa, Panama. Butterflies were collected from the field (collection permit no. SE/A-5-20; Supplementary Table 4.1) or taken from the insectaries in Gamboa. Butterflies reared in the insectaries were raised under natural conditions with their host plants, and adults fed a pollen/sugar solution daily. Wild butterflies caught from the field were held in the insectaries for 1 – 2 days before trials. This ensured both wild and insectary-reared butterflies experienced the same conditions for a few days before the trials.

All trials were undertaken between 8:45 – 16:20 in ambient conditions (ca. 26 °C, 60 % humidity). Butterflies were tested one at a time. Before the trial, the butterfly was put in an envelope in the fridge for four minutes at ca. 6 – 8 °C. It was then removed from the fridge and the envelope and placed on a white cardboard stand inside an aluminium lined box (55 x 35 x 35 cm). The box was opened at the front and back to prevent heat from being trapped and heating the butterfly during the trial. Within 10 seconds before the butterfly was placed on the stand, a 125W lamp was placed 16 cm above the cardboard stand (Supplementary Fig. 4.1). This prevented the stand and the air inside the box from heating before the butterfly was placed on the stand and ensured that the air inside the box was close to ambient temperature. The amount of radiation to reach the cardboard stand, and hence the butterfly, was 419 W/m². Although this radiation level is lower than what butterflies experience on a clear day in tropical regions (ca. 1200W/m²), it is comparable to other studies and provides enough radiation to test butterfly take-off temperature and heating rate (Tsai et al., 2020). The butterfly was recorded with a thermal camera (FLIR A700-EST, Teledyne FLIR, Oregon, United States) from the moment it was placed on the stand until it flew off the stand. The stand and box were allowed to cool to ambient temperature between trials. After each trial, the butterfly's forewing length (mm) and thorax diameter (mm) were measured and its wing loading and wing aspect ratio were calculated. Its condition was scored, as above. Only undamaged butterflies (score 1-3) were used in trials.

The above laboratory study was repeated in the Czech Republic, under the same conditions (ca. 26 °C, 60 % humidity), with some minor modifications using dead, dried, pinned butterfly specimens. The specimens were removed from their pin before each trial and placed, suspended on hairs, above the cardboard stand. Butterflies were recorded with a thermal camera (WIRIS 2nd Gen, Workswell, Prague, Czech Republic) for a total of 2 mins 30 secs.

4.3.3 Take-off temperature and heating rate video analyses

Thermal videos were analysed using FLIR Research Studio (Teledyne FLIR, Oregon, United States) and Workswell CorePlayer (Workswell, Prague, Czech Republic) software. Emissivity was set to 0.95, room temperature 26 °C, reflected temperature 26 °C, distance 0.2 m and humidity 60% (Advani et al., 2019; Mattila, 2015). T_b of the butterfly was calculated as the

mean of a square in the centre of the thorax. Take-off temperature, for the live butterfly trials, was determined as T_b just before the butterfly flew off the cardboard stand. For heating rate ($^{\circ}\text{C min}^{-1}$) of live and dead butterflies, the difference between T_b at zero and 60 seconds was calculated (Wang et al., 2021). Body temperature at zero seconds was measured just after the butterfly was placed on the cardboard stand under the lamp. For live butterfly trials, any trial where the butterfly's thorax was not clearly visible, or where the butterfly walked off the cardboard stand, were excluded from analysis. For take-off temperature, the trial was also excluded from analysis if the butterfly had not taken-off after three minutes. For live butterfly trials, only species/subspecies with at least five take-off temperature or live heating rate measurements per sex were included in the analysis. For dead butterfly trials, only species/subspecies with at least three heating rate measurements were included in the analysis.

4.3.4 Phylogeny and morphological traits

Wing colour lightness was assessed visually as the percentage of black on both hind and forewing for each species/subspecies. Species/subspecies were classified as either having iridescence on their wings or not and were assigned to a habitat type (i. closed canopy lowland forest, ii. lowland forest edges and gaps, iii. mixed lowland habitats, iv. high-altitude habitat) according to the literature (Benson, 1978; Descimon & Mast, 1984; Estrada & Jiggins, 2002; Montejo-Kovacevich et al., 2020; Reed, 2003; Tobler et al., 2005). The *Heliconius* phylogeny was obtained from Cicconardi *et al.* (2023) and pruned to the species/subspecies present in our data (buffering ability $n = 12$; take-off temperature $n = 12$; live heating rate $n = 10$; dead heating rate $n = 16$). Subspecies which were not present in the phylogeny (*H. erato hydara* and *notabilis*, and *H. melpomene ecuadorensis*, *malleti* and *plesseni*) were added based on previously established subspecies relationships with inserted branches having half the original parent branch length (Nadeau et al., 2014). The three *H. numata* morphotypes were added as a polytomy at the tip of the *H. numata* branch with very short branch lengths (0.01) to represent the within-population polymorphism in this species (Joron et al., 2006).

4.3.5 Data analyses

All statistical analyses were undertaken in R version 4.1.2 (R Core Team, 2022). Graphics were produced using the package *ggplot2* (Wickham, 2016). Where necessary mean wing length was square root transformed to normalise data. To ensure normality and homogeneity in the residuals, graphical model diagnostics were applied to each model.

Buffering ability

To ensure that our estimates of buffering ability were robust, only species/subspecies with at least ten T_b measurements were included in the analysis. Simple linear regressions with T_b as the response variable and T_a as the predictor variable were fitted separately for each species/subspecies to estimate their buffering ability. The slope of the relationship was extracted and subtracted from one to provide an estimate of buffering ability (Bladon et al. 2020). With this method, a steep slope shows that the butterfly's T_b varies greatly over small ranges of T_a , thus indicating a low buffering ability and a negative buffering ability estimate. A shallow slope shows that the butterfly's T_b remains relatively stable over wide ranges of T_a thus indicating a high buffering ability and a positive buffering ability estimate.

Does morphology and relatedness drive butterfly thermoregulation?

To examine drivers of buffering ability, linear mixed effects models (LMM) were fitted using the R package *lmer4* (Bates et al., 2015). Mean forewing length and colour lightness were strongly correlated, however model diagnostics showed we had good coverage of both these predictor variables along the entire T_a range and so we kept both variables in the model. No other continuous model predictors were highly correlated (Supplementary Fig. 4.2). T_b was fitted as the response variable. T_a , sex, condition, mean forewing length, mean wing load, mean forewing aspect ratio and iridescence and the two-way interactions between T_a and each of the other terms were predictor variables. Species/subspecies identity was included as a random effect. To find the best-fit model, we used backwards stepwise selection with the step function in R package *lmerTest* (Kuznetsova et al., 2017). We re-ran best-fit models using phylogenetic generalised linear mixed models (PGLMM) using the R package *phyr* (Li et al.,

2020) to account for evolutionary relationships in these models. The pruned phylogeny of *Heliconius* butterflies, described above, was used to fit the PGLMM using a Bayesian framework (Cicconardi et al., 2023). Goodness of fit was measured using the R package *rr2* (Ives & Li, 2018) for PGLMMs. The PGLMMs are presented in the results and the LMMs in the supplementary material. Marginal means were predicted using the package *ggeffect* (Lüdtke, 2018) and the difference between marginal means across 20 °C T_a was used as a measure of effect size.

To examine drivers of take-off temperature and heating rate LMMs were fitted. Take-off temperature and heating rate were fitted separately as response variables and sex, condition, forewing length, wing load, forewing aspect ratio, colour lightness and iridescence as predictor variables. Species/subspecies identity was included as a random effect. To find the best-fit model we used backwards stepwise selection (as described above). The best-fit models were re-ran using PGLMMs (as described above) to account for potential effects of evolutionary history in these models and to test for an overall phylogenetic effect. Pearson correlation test showed that none of the continuous model predictors were highly correlated (Supplementary Fig. 4.3-4.5).

To test if mimicry affects differ in buffering ability, take-off temperature and heating rate data were subset to only include the mimetic pairs and PGLMMs were fitted. Each mimetic pair was given an ID (pair ID). For buffering ability, buffering ability was fitted as the response variable, and T_a and pair ID and the interaction between T_a and pair ID were fitted as predictor variables. Species/subspecies identity was included as a phylogenetic and non-phylogenetic random term. For take-off temperature and heating rate, take-off temperature and heating rate were fitted separately as response variables and only pair ID was fitted as the predictor variable.

To test for differences in buffering ability, take-off temperature and heating rate within mimetic pairs, a simple linear regression was fitted for each mimetic pair separately. T_b , take-off temperature and heating rate were fitted separately as response variables. For buffering ability, T_a and species/subspecies and the two-way interaction between T_a and species/subspecies were fitted as predictor variables. An F-test was performed to determine

if the model with the interaction term was a better fit for the data than the model without the term; if so, it would suggest that the species/subspecies within the mimetic pair differed in their buffering ability. For take-off temperature and heating rate subspecies only was fitted as a predictor variable and this model was compared using an F-test to a null model.

Do butterflies from different habitats differ in thermoregulatory ability?

To test for differences in buffering ability, take-off temperature and heating rate of butterflies from different habitat types, a PGLMM was fitted to the data. T_b , take-off temperature and heating rate were fitted separately as response variables. For buffering ability, T_a and habitat type and the two-way interaction between T_a and habitat type were fitted as predictor variables. We included species/subspecies identity as a random effect and accounted for phylogenetic signal. For take-off temperature and heating rate, habitat type only was fitted as the predictor variable. There was only one high altitude species (*H. clysonimus*) sampled in the field study and the T_a at which it was measured was very different to that at which the other species/subspecies were sampled. Therefore, this species was removed from the analysis of buffering ability estimate. There was one high altitude species (*H. himera*) used in the take-off temperature experiments. This species was retained in the analysis, and the results were interpreted considering this. To control for differences in T_a across different habitat types the model with T_b as the response variable was repeated on a subset dataset where T_a was restricted to the same range across habitat types.

4.4 Results

4.4.1 Buffering ability

Overall, 424 individuals from 13 species/subspecies were measured in the field in Panama. Body temperature (T_b) ranged from 19.3 °C to 36.0 °C, with a mean of 28.8 °C. Air temperature (T_a) ranged from 15.7 °C to 32.8 °C, with a mean of 25.9 °C. The buffering ability estimate ranged from -0.317 (*H. erato hydara*) to 0.564 (*H. ethilla claudia*), with *H. ethilla claudia* better able to regulate their T_b in response to changes in T_a than *H. erato hydara* (Supplementary Table 4.2; Supplementary Fig. 4.6). The best LMM of buffering ability included T_a , wing aspect

ratio, wing load, colour lightness, the interaction between T_a and colour lightness and the random effect of species/subspecies identity (PGLMM partial $R^2 = 0.72$; Supplementary Table 4.3a and 4.4a). Colour lightness was the main driver of buffering ability with lighter species/subspecies being better at buffering their T_b against changes in T_a than darker species/subspecies (Estimate = 0.086, 95% CI = 0.029 – 0.143; Supplementary Table 4.4a; Fig. 4.3). In line with our hypothesis, phylogeny accounted for a small proportion of the variance (<0.001%) in our model showing that buffering ability is not phylogenetically constrained (Supplementary Table 4.4a).

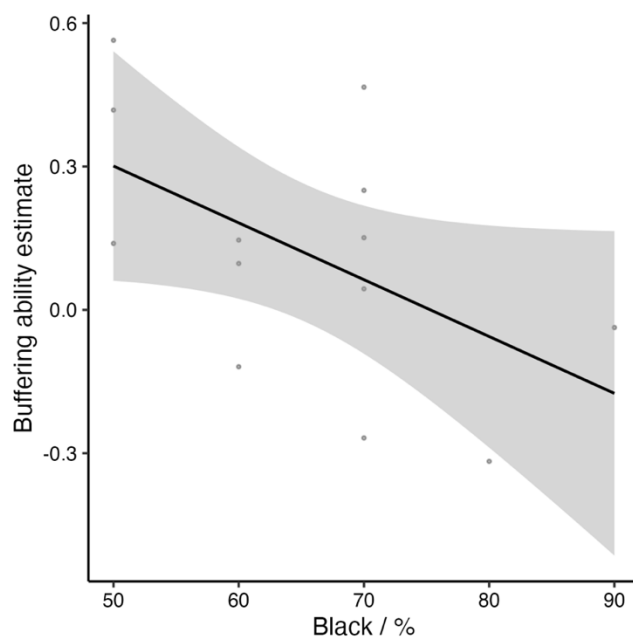


FIGURE 4.3. Mean buffering ability estimate and colour lightness, estimated as percentage of black on both hind and forewing. Each point represents a species/subspecies. The grey band shows the 95% confidence interval.

There were no differences between any of the four mimetic pairs in their buffering ability (Table 4.1a). When comparing species/subspecies within mimetic pairs, only subspecies within one mimetic pair differed in their buffering ability: *H. melpomene rosina* were better at buffering their T_b against changes in T_a than *H. erato demophoon* ($F = 6.190$, $df = 1$, $p = 0.014$) (Supplementary Table 4.5; Fig. 4.4). This means that across a 20.0 °C range of T_a , predicted *H. melpomene rosina* T_b would vary by 10.7 °C, whereas predicted *H. erato demophoon* T_b would vary by 19.1 °C. There were no significant differences in the buffering ability estimate between species/subspecies within the other mimetic pairs (Supplementary Table 4.5; Fig. 4.4).

TABLE 4.1. Model output for phylogenetic generalised linear mixed models (PGLMMs) of the effect of pair number on a. buffering ability, b. take-off temperature, c. live heating rate and d. dead heating rate. Bold indicates significant effects; coefficients where the 95% Bayesian credible interval (CI) does not overlap zero.

a				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.007	<0.001	<0.001
1 sp__	<0.001	0.007	<0.001	<0.001
residual	1.858	1.363	1.563	2.225
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	9.394	3.898	14.889	
Tair	0.758	0.545	0.970	
Pair.no.2	-3.131	-9.538	3.280	
Pair.no.3	-0.830	-11.321	9.662	
Pair.no.4	-1.039	-9.333	7.260	
Tair:Pair.no.2	0.107	-0.139	0.354	
Tair:Pair.no.3	0.018	-0.384	0.421	
Tair:Pair.no.4	0.020	-0.306	0.347	
b				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.008	<0.001	<0.001
1 sp__	<0.001	0.006	<0.001	<0.001
residual	15.970	3.996	13.640	18.830
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	37.141	36.062	38.220	
Pair.no.2	-1.685	-3.080	-0.290	
Pair.no.3	-2.746	-4.488	-1.004	
Pair.no.4	-3.344	-5.121	-1.568	
Pair.no.5	-2.108	-3.835	-0.382	
Pair.no.6	0.871	-0.944	2.686	
Pair.no.7	-0.267	-1.859	1.326	
c				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.007	<0.001	0.001
1 sp__	<0.001	0.007	<0.001	0.001
residual	4.609	2.147	3.724	5.811
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	11.465	10.827	12.104	
Pair.no.2	1.939	1.042	2.837	
Pair.no.3	-0.043	-1.204	1.119	
Pair.no.5	1.156	-0.028	2.340	
Pair.no.7	1.477	0.510	2.443	
d				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.007	<0.001	<0.001
1 sp__	<0.001	0.007	<0.001	<0.001
residual	2.683	1.638	1.938	3.932

Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	17.399	16.419	18.380	
Pair.no.2	-1.279	-2.700	0.142	
Pair.no.3	-0.562	-1.949	0.825	
Pair.no.4	0.334	-1.128	1.796	
Pair.no.5	-0.541	-1.898	0.817	
Pair.no.6	0.676	-0.682	2.033	

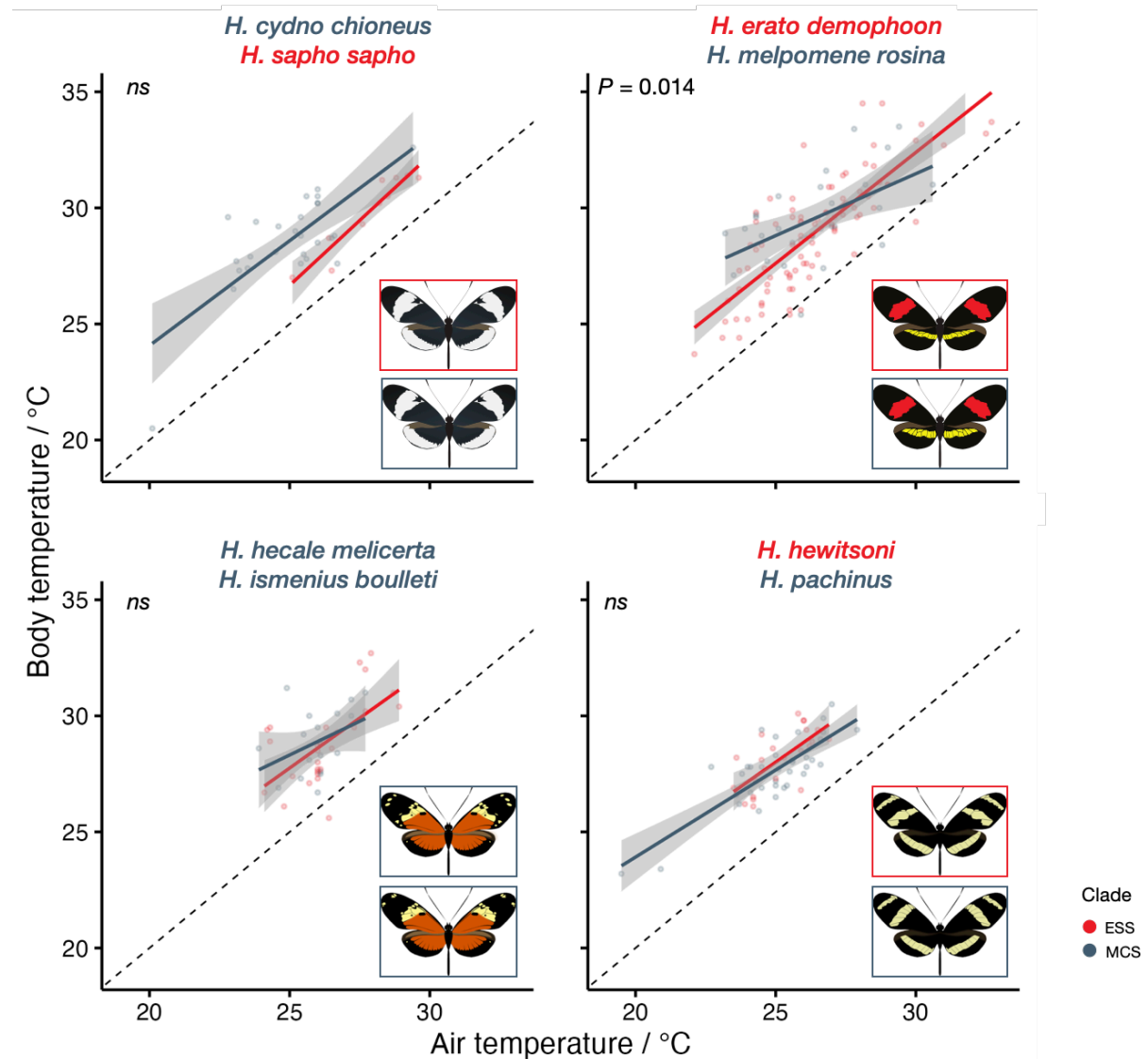


FIGURE 4.4. Individual butterfly body temperatures (°C) at different air temperatures (°C) of mimetic *Heliconius* pairs. Solid lines represent the modelled relationship between body temperature and air temperature. The grey bands show 95% confidence intervals. The black dashed line represents a 1:1 relationship between body and air temperature. Significant p-values are provided for likelihood ratio tests showing a significant difference in buffering ability estimate between butterflies within a pair. A non-significant (ns) difference in buffering ability estimate between butterflies with a pair is also marked. The *Erato/Sara/Sapho* clade (ESS) is in red and the *Melpomene/Cydno/Sylvaniform* clade (MCS) in blue. Butterfly illustration credit: Steven Van Belleghem.

T_b of closed canopy species/subspecies ranged from 20.5 °C to 32.7 °C (mean 29.3 °C), of forest edge and gap species/subspecies ranged from 23.7 °C to 35.9 °C (mean 29.3 °C) and species/subspecies from mixed habitats ranged from 20.9 °C to 36.0 °C (mean 29.0 °C). In the unrestricted dataset, where T_a was not restricted to the same range across all habitat types, butterflies from different habitat types did not respond differently to T_a (Supplementary Table 4.6a and 4.7; Fig. 4.5a). In the restricted dataset, where T_a was restricted to the same range across all habitat types, closed canopy forest species/subspecies (buffering ability estimate 0.44 ± 0.03) were better at buffering than species/subspecies from forest edge and gaps (buffering ability estimate 0.03 ± 0.07) and mixed habitat (buffering ability estimate 0.09 ± 0.06 ; Supplementary Table 4.8a; Supplementary Fig. 4.7). Phylogeny explained <0.001% of the variance in both the unrestricted and restricted dataset, indicating that differences in buffering ability between habitat types was not due to relatedness (Supplementary Table 4.6a and 4.8a).

4.4.3 Take-off temperature

Take-off temperature was measured on 18 species/subspecies ($n = 354$), including seven mimetic pairs and ranged from 29.5 °C to 38.5 °C with a mean of 34.8 °C (Supplementary Table 4.2). The best LMM only included species/subspecies identity as the random factor (Supplementary Table 4.3b). The PGLMM showed that phylogeny explained <0.001% of the variance indicating that any differences in take-off temperature between species/subspecies were not due to relatedness (partial $R^2 = 0.19$; Supplementary Table 4.4b). Take-off temperature differed between mimetic pairs: the *H. erato notabilis* and *H. melpomene plesseni* pair took off at a higher temperature (38.0 ± 0.8 °C) than the *H. hewitsoni* and *H. pachinus* pair (33.8 ± 0.6 °C) (Table 4.1b). When comparing take-off temperature of butterflies within mimetic pairs, *H. cydno chioneus* (34.1 ± 1.1 °C) took off at a significantly lower temperature than *H. sapho sapho* (38.5 ± 0.5 °C) ($F = 20.121$, $df = 1$, $p < 0.001$). There were no other differences in take-off temperatures within the other mimetic pairs tested (Supplementary Table 4.5). The high-altitude species *H. himera* (29.5 ± 2.0 °C) took off at a significantly lower temperature than butterflies from other habitats (Estimate = -5.086, 95% CI = -9.589 to -0.537; mixed: 34.1 ± 0.5 °C; closed canopy: 35.5 ± 0.5 °C; edge and gaps: 36.1 ± 0.3 °C) and phylogeny explained 0.001% of the variance (Supplementary Table 4.6b and 4.7;

Fig. 4.5b). When undertaking the analysis without the one high-altitude species, *H. himera*, there was no longer a significant effect of habitat type on take-off temperature and phylogeny explained more of the variance (0.034%; Supplementary Table 4.8b).

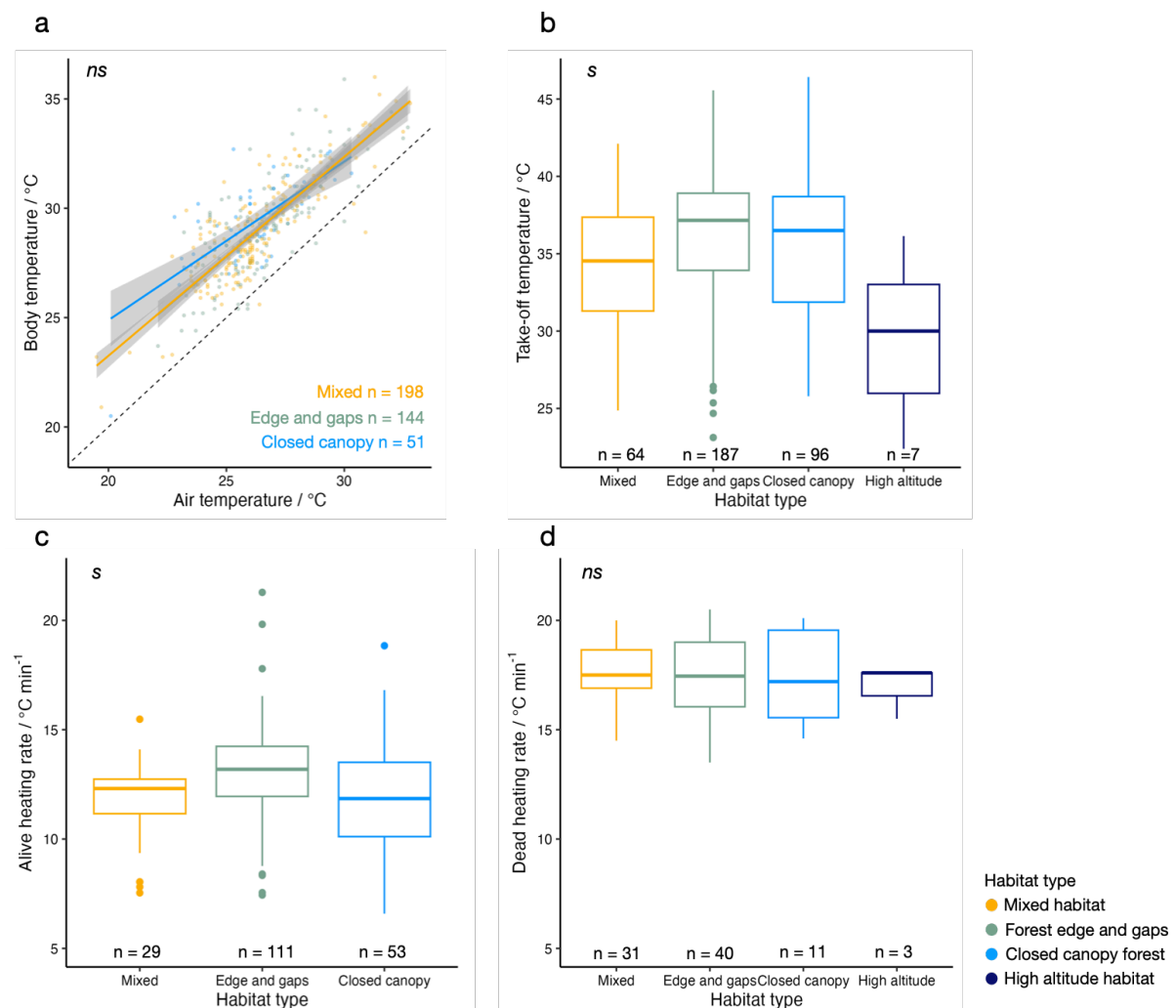


FIGURE 4.5. **a.** Butterfly body temperatures (°C) at different air temperatures (°C), **b.** Take-off temperature (°C), **c.** heating rate of live butterflies (°C min⁻¹) and **d.** heating rate of dead butterflies (°C min⁻¹) from different habitats: mixed (yellow), edge and gaps (green), closed canopy (light blue) and high altitude (dark blue). In **a.** solid lines represent the modelled relationship between body temperature and air temperature, the grey bands show 95% confidence intervals, and the black dashed line represents a 1:1 relationship between body and air temperature. A non-significant (ns) or significant (s) difference in buffering ability, take-off temperature and heating rate of live and dead butterflies between butterflies from different habitats is marked.

4.4.4 Heating rate of live butterflies

Heating rate of live butterflies was measured on 13 species/subspecies (n = 193), including five mimetic pairs and ranged from 10.8 °C min⁻¹ to 14.5 °C min⁻¹ with a mean of 12.6 °C min⁻¹.

¹ (Supplementary Table 4.2). The best LMM included forewing length and iridescence and both terms were significant in the PGLMM (Iridescence estimate = -1.395, 95% CI = -2.042 to -0.748; Forewing length estimate = -2.410, 95% CI = -3.309 to -1.512; partial R^2 = 0.18; Supplementary Table 4.3c and 4.4c). Phylogeny explained <0.001% of the variance indicating that relatedness did not determine butterfly heating rate (Supplementary Table 4.4c). Butterflies with small forewing lengths had a higher heating rate than butterflies with large forewing lengths. Iridescent species/subspecies had a lower heating rate than non-iridescent species/subspecies (iridescent: 11.6 ± 0.2 °C min⁻¹; non-iridescent: 12.9 ± 0.3 °C min⁻¹). There was a significant effect of mimetic pair ID on heating rate of live butterflies with the *H. erato demophoon* and *H. melpomene rosina* pair heating at a greater rate (13.4 ± 0.3 °C min⁻¹) than the *H. hecale melicerta* and *H. ismenious bouletti* pair (11.4 ± 0.6 °C min⁻¹; Table 4.1c). Within mimetic pairs, *H. erato etylus* (10.8 ± 1.1 °C min⁻¹) had a significantly lower heating rate than *H. melpomene ecuadorensis* (13.3 ± 0.3 °C min⁻¹) ($F = 9.489$, $df = 1$, $p = 0.007$) and *H. melpomene malletti* (12.6 ± 0.4 °C min⁻¹) had a significantly lower heating rate than *H. erato lativitta* (14.5 ± 0.9 °C min⁻¹) ($F = 4.010$, $df = 1$, $p = 0.054$). There were no other differences in heating rate within the other three mimetic pairs tested (Supplementary Table 4.5). Forest edge and gap butterflies (13.0 ± 0.2 °C min⁻¹) heated up at a quicker rate than those from closed canopy (11.9 ± 0.3 °C min⁻¹) or mixed habitats (11.7 ± 0.4 °C min⁻¹) (Estimate = 1.128, 95% CI = 0.411 – 1.845; Supplementary Table 4.6c and 4.7; Fig. 4.5c). Phylogeny explained <0.001% of the variance, indicating that relatedness did not affect live heating rate (Supplementary Table 4.6c).

4.4.5 Heating rate of dead butterflies

Heating rate of dead butterflies was measured on 16 species/subspecies ($n = 85$), including six mimetic pairs and ranged from 13.5 °C min⁻¹ to 20.5 °C min⁻¹ with a mean of 17.44 °C min⁻¹ (Supplementary Table 4.2). The best LMM model included forewing length and colour lightness and both terms were significant in the PGLMM (Forewing length estimate = 0.203, 95% CI = 0.071 – 0.334; Colour lightness estimate = 0.609, 95% CI = 0.306 – 0.911; partial R^2 = 0.17; Supplementary Table 4.3d and 4.4d). Phylogeny explained <0.001% of the variance, indicating that relatedness did not affect dead heating rate (Supplementary Table 4.4d). Butterflies with small forewing lengths had a lower heating rate than butterflies with large

forewing lengths and lighter coloured butterflies had a lower heating rate than darker butterflies. There was no significant effect of mimetic pair ID on heating rate of dead butterflies (Table 4.1d). There was no difference in the heating rate of species/subspecies within the mimetic pairs tested (Supplementary Table 4.5). Butterflies from different habitats did not heat up at different rates and phylogeny again explained <0.001% of the variance in this model (Supplementary Table 4.6d and 4.7; Fig. 4.5d).

4.5 Discussion

To our knowledge this is the first study which uses Müllerian mimics to test the effects of morphology and relatedness on species thermoregulatory abilities. Overall, we found that morphological traits, not relatedness, were important in determining *Heliconius* thermoregulatory abilities. Colour, iridescence, and size were all important for *Heliconius* thermoregulation. Light butterflies were better at buffering their body temperature against changes in air temperature and when dead, heated up at a lower rate than dark butterflies. This result is supported by other studies which found that light coloured butterflies effectively thermoregulate by reflecting solar radiation onto or away from their thorax and heat up at a lower rate than dark butterflies (Bladon et al., 2020; Kingsolver, 1988; Shanks et al., 2015). We found that iridescent butterflies, when alive, heated up at a lower rate than non-iridescent butterflies. Iridescence, which is caused by nanostructures on the surface of the wing scales, is linked to thermoregulation via radiative cooling (Tang et al., 2022; Tsai et al., 2020). In particular, *H. sara*, one of our focal species, was recently found to heat up less than other butterfly species, due to the high visible reflectance and infrared emittance properties of its wings (Krishna et al., 2020).

The morphological traits driving heating rate differed between dead and live butterflies indicating that behaviour and/or physiology also determine *Heliconius* thermoregulation. When alive, large and iridescent butterflies had a lower heating rate than small and non-iridescent butterflies. However, when dead, small and light butterflies had a lower heating rate than large and dark butterflies. This difference could be due to behavioural or physiological mechanisms. Butterflies can sense visible and infrared radiation via their wings, allowing them to respond behaviourally to prevent overheating (Tsai et al., 2020). Large,

iridescent wings may only help with thermoregulation and cooling when coupled with behavioural positioning of the wings to reflect solar radiation away from the thorax (Kemp & Krockenberger, 2002; Shanks et al., 2015; Tsai et al., 2020). Alternatively, in dead butterflies, where thermoregulatory behaviour is not exhibited, large and dark individuals heat up faster as the larger surface area and dark colour results in greater absorption of solar radiation (Kingsolver, 1988). This result highlights the importance of undertaking thermal physiology experiments on live specimens, thereby ensuring behavioural and physiological mechanisms, in addition to morphological traits, are investigated and ecologically relevant data is obtained.

Although relatedness did not drive thermoregulatory ability, and distantly related butterflies within mimetic pairs mostly did not differ in buffering ability, take-off temperature and heating rate, there were some important exceptions. For example, *H. erato demophoon* and *H. melpomene rosina* differed in their buffering ability, with *H. melpomene rosina* being better at buffering than *H. erato demophoon*. This result was likely explained by size differences between the co-mimics, with the larger *H. melpomene rosina* being better at buffering. This observation is in line with another study which found large butterflies are better at buffering than small butterflies (Bladon et al., 2020). Take-off temperature also differed between the two co-mimetics *H. cydno chioneus* and *H. sapho sapho*, with *H. cydno chioneus* taking off at a lower temperature than *H. sapho sapho*. Similarly, heating rate of live butterflies differed between *H. erato etylus* and *H. melpomene ecuadorensis*, and *H. melpomene malletti* and *H. erato lativitta*, but the differences between species were not consistent, with *H. erato etylus* and *H. melpomene malletti* having a lower heating rate than *H. melpomene ecuadorensis* and *H. erato lativitta* respectively. Here, size differences were not obviously driving these observations. Rather, similar to the differences seen in heating rate of live and dead butterflies, the difference in thermoregulatory ability may be due to species-specific behavioural or physiological mechanisms. Despite these exceptions, across the entire dataset, there was a significant effect of pair ID on take-off temperatures and heating rate of live butterflies. Thus, distantly related co-mimics which share near identical wing patterns had similar thermoregulatory abilities underscoring the strong effect of morphology across the lineage.

4.5.1 Habitat specialisation

Our data included species found in different habitats (mixed habitat, forest edge and gaps, and closed canopy forest) and occurring at different altitudes (low- and mid-elevation). Unlike the clear signal of morphology on thermoregulatory abilities, the habitat associated differences in thermoregulation across the species and subspecies we examined were more nuanced. Indeed, in the unrestricted data set, we found there was no difference in buffering ability between butterflies from different habitats. Nonetheless, when we restricted the dataset to the same range of air temperatures across habitat types there was evidence that butterflies from different habitats differed in their thermoregulatory abilities. Thus, our data supported the hypothesis that species are adapted or acclimatised to the specific thermal environment they inhabit. However, the differences were not always in line with our expectations. For example, contrary to our expectation, butterflies from hotter environments (forest edges and gaps) were not better at buffering than butterflies found predominately in closed canopy habitats. This was partially driven by closed canopy butterflies raising their body temperature at low air temperatures. This could be an adaptation to cool forest interiors, allowing the butterflies to become more active at low air temperatures and may be explained by the colouration of the closed canopy butterflies we studied. Both *H. cydno chioneus* and *H. sapho sapho* have a large amount of white on the wings, which being highly visible against dense forest, is thought to act as a warning signal to predators and/or a mating signal in the dark forest understory (Sweeney et al., 2003). These white patches may also act as solar concentrators, reflecting what little solar radiation reaches the forest understory onto the butterfly's thorax, enabling the butterfly to raise its temperature at low air temperatures and thereby maintain a more stable body temperature (Shanks et al., 2015). In addition, closed canopy butterflies lowered their body temperature at high air temperatures to a greater extent than butterflies from forest edges and gaps and mix habitat. This ability was likely achieved by utilising the cool microclimates most frequently found in forest interiors (Pincebourde & Woods, 2020). This result is similar to a previous study which showed that woodland butterflies kept a lower body temperature than open grassland butterflies (Kleckova & Klecka, 2016). We found that forest edge and gap and mixed habitat butterflies did not maintain such stable body temperatures over a range of air temperatures.

In live butterflies, forest edge and gap butterflies heated up at a faster rate than those from mixed habitat and closed canopy forest. This is contrary to what we hypothesised, that to avoid overheating, forest edge and gap butterflies would heat up at a slower rate compared those from closed canopy forests. We would have expected closed canopy forest butterflies to heat up at a higher rate to maximise flight time at the lower air temperatures of forest interiors. Heating rate has been shown to positively correlate with take-off temperature, and forest edge and gaps butterflies had the highest take-off temperature than butterflies from other habitats (Neve & Hall, 2016). This may explain why forest edge and gap butterflies had a faster heating rate.

There was also some evidence for thermal acclimatisation/adaptation to different altitudes. Specifically, the high-altitude species *H. himera*, took off at lower temperatures than butterflies from lower elevation. This finding was consistent with earlier behavioural work that compared daily activity patterns of *H. himera* and *H. erato cyrbia* and is also in line with other studies which found high-altitude butterfly populations took off at lower air temperatures than low-altitude populations (Advani et al., 2019; Davison et al., 1999; Kingsolver, 1983; MacLean et al., 2016). This may be an adaptation to the greater solar radiation a butterfly is exposed to at high altitudes (Bonebrake et al., 2014). When flying in full sun, butterflies by receiving higher solar radiation, may not lose a lot of heat during flight and can therefore take-off at lower temperatures. Alternatively, it may be that high altitude butterflies have adapted to fly at low air temperatures, in an effort to avoid spending large amounts of time basking to raise their body temperature.

4.5.2 Significance and Conclusions

Our results show that individual species have unique thermoregulatory abilities as a consequence of their morphology, behaviour, physiology and local thermal environment. As such, our study underscores the need to collect ecologically relevant data on live butterflies to further our understanding of species vulnerabilities to climate change and habitat fragmentation. This study also highlights the need for more focal studies of the thermoregulatory abilities of tropical insects. Over 85% of the world's insects live in tropical regions; yet, most of our knowledge of ectotherms comes from temperate regions (Bonebrake

et al., 2010; Stork, 2018). If we hope to understand the overall effects of climate change on species and community composition, we need more data on how tropical species cope with environmental stress. Moreover, we need to extend this research to other life stages. Here, we focus only on the adult stage. Although our data suggest that light-coloured, iridescent and large butterflies may be able to cope better with moderate climate warming than dark-coloured, non-iridescent and small butterflies, what happens to the egg, larvae, and pupae during environmental stress may be as important, or more important, for understanding a species response to climate change (Kingsolver et al., 2011).

4.6 Bibliography

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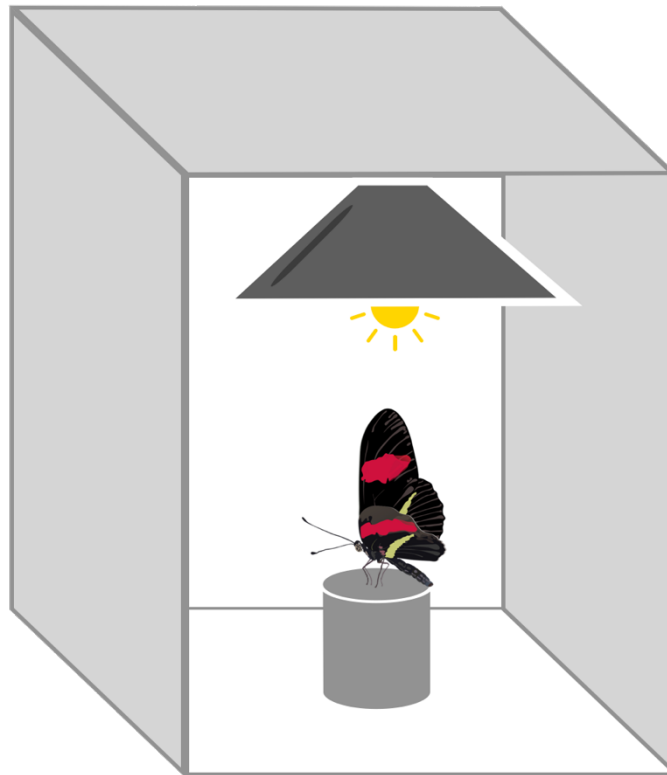
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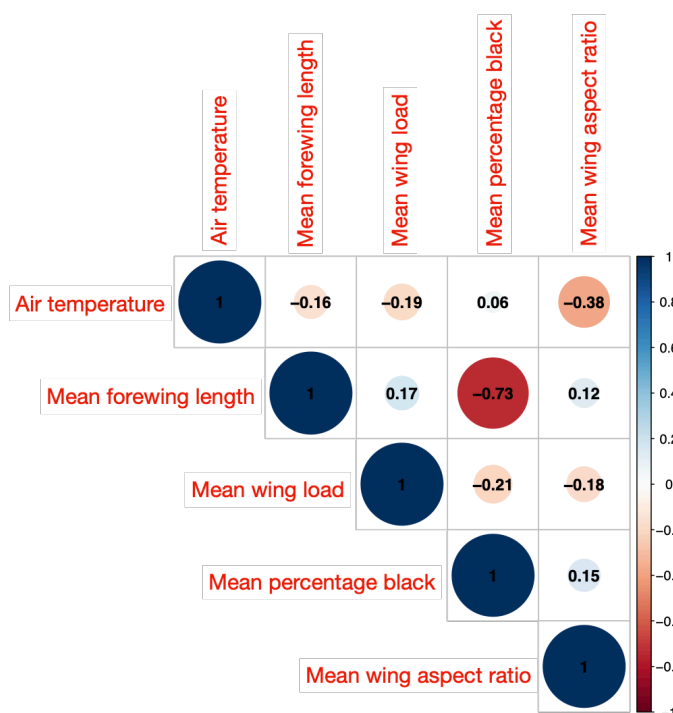
4.7 Supplementary material

SUPPLEMENTARY TABLE 4.1. *Study sites in Panama, including coordinates, elevation (meters above sea level) and land type.*

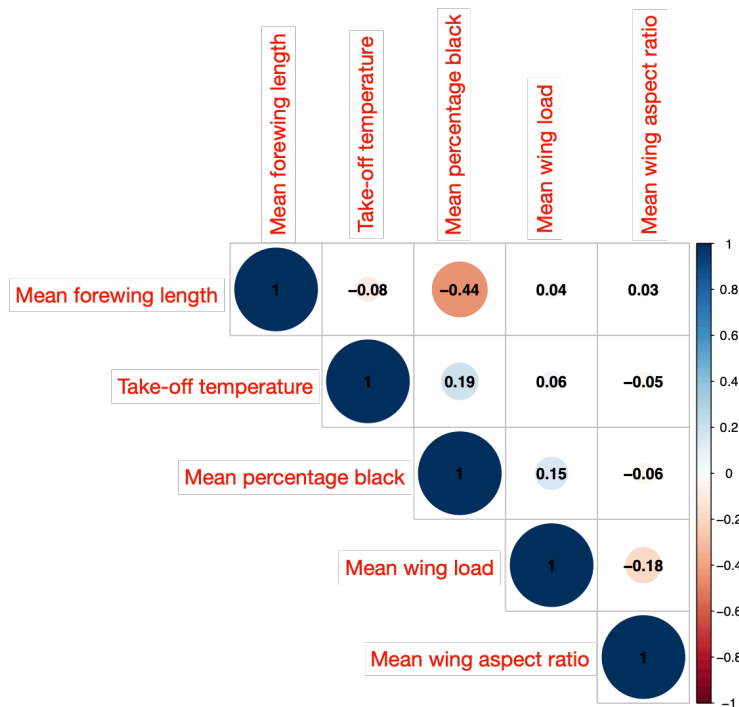
Site	Coordinates		Elevation	Land type
	N	E		
Achiote	9.245165	-80.025921	91	Lowland tropical moist encroaching scrub
Cerro Azul	9.212178	-79.41811	760	Pre-montane tropical wet encroaching scrub and secondary forest
El Valle	8.617975	-80.115683	735	Lowland tropical wet encroaching scrub
Gamboa	9.116637	-79.696492	50	Lowland tropical managed urban green spaces
La Laguna Volcán	8.769125	-82.673225	1355	Pre-montane tropical wet encroaching scrub and secondary forest
Las Minas	7.753728	-80.767706	528	Lowland tropical wet encroaching scrub and secondary forest
Los Pozos	8.863298	-82.694739	1658	Pre-montane tropical wet encroaching scrub and secondary forest
Metetí	8.630044	-77.954936	31	Lowland tropical moist encroaching scrub and secondary forest
Mount Totumas	8.882948	-82.683699	1880	Lower mountain rain forest and managed agroforestry sites
Parque Nacional Altos de Campana	8.705518	-79.887839	284	Premontane wet encroaching scrub and secondary forest
Parque Nacional San Lorenzo	9.246993	-79.978955	177	Secondary lowland tropical wet forest
Parque Nacional Soberanía	9.125578	-79.714579	84	Secondary semi-deciduous lowland tropical wet forest
Sajalices	8.680064	-79.880754	98	Lowland tropical wet encroaching scrub and secondary forest
San Carlos	8.624428	-80.048144	858	Lowland tropical moist encroaching scrub and secondary forest
Santa Rita Arriba	9.353508	-79.752644	253	Lowland tropical moist encroaching scrub
Volcán	8.653233	-82.544062	832	Pre-montane tropical wet encroaching scrub and secondary forest



SUPPLEMENTARY FIGURE 4.1. Schematic of experimental set up for undertaking measurements of take-off temperature and heating rate. Butterfly illustration credit: Steven Von Belleghem.



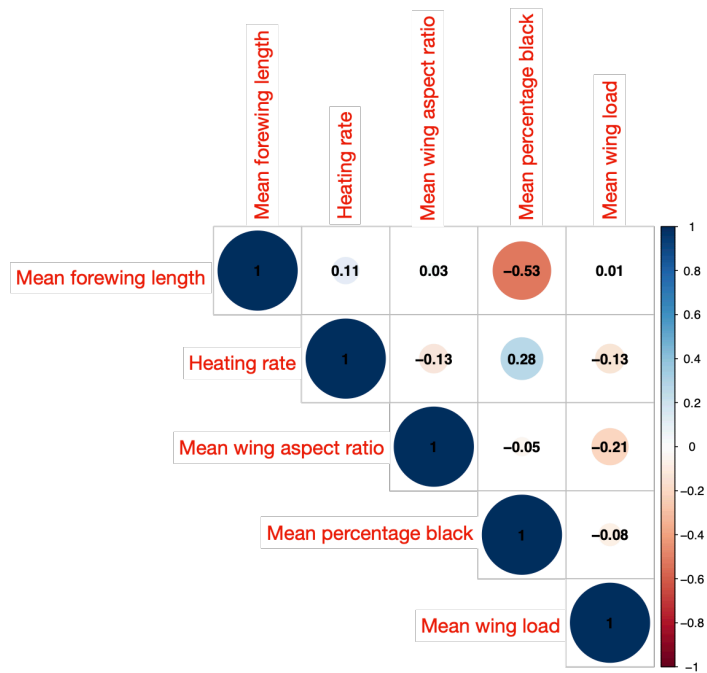
SUPPLEMENTARY FIGURE 4.2. Correlation among continuous predictor variables from the buffering ability dataset. Correlation between the mean percentage of black on the fore- and hindwings, a proxy for colour lightness, and mean forewing length (mm) is high.



SUPPLEMENTARY FIGURE 4.3. Correlation among continuous predictor variables from the take-off temperature dataset. Correlation between the percentage of black on the fore- and hindwings, a proxy for colour lightness, and mean forewing length(mm) is medium.



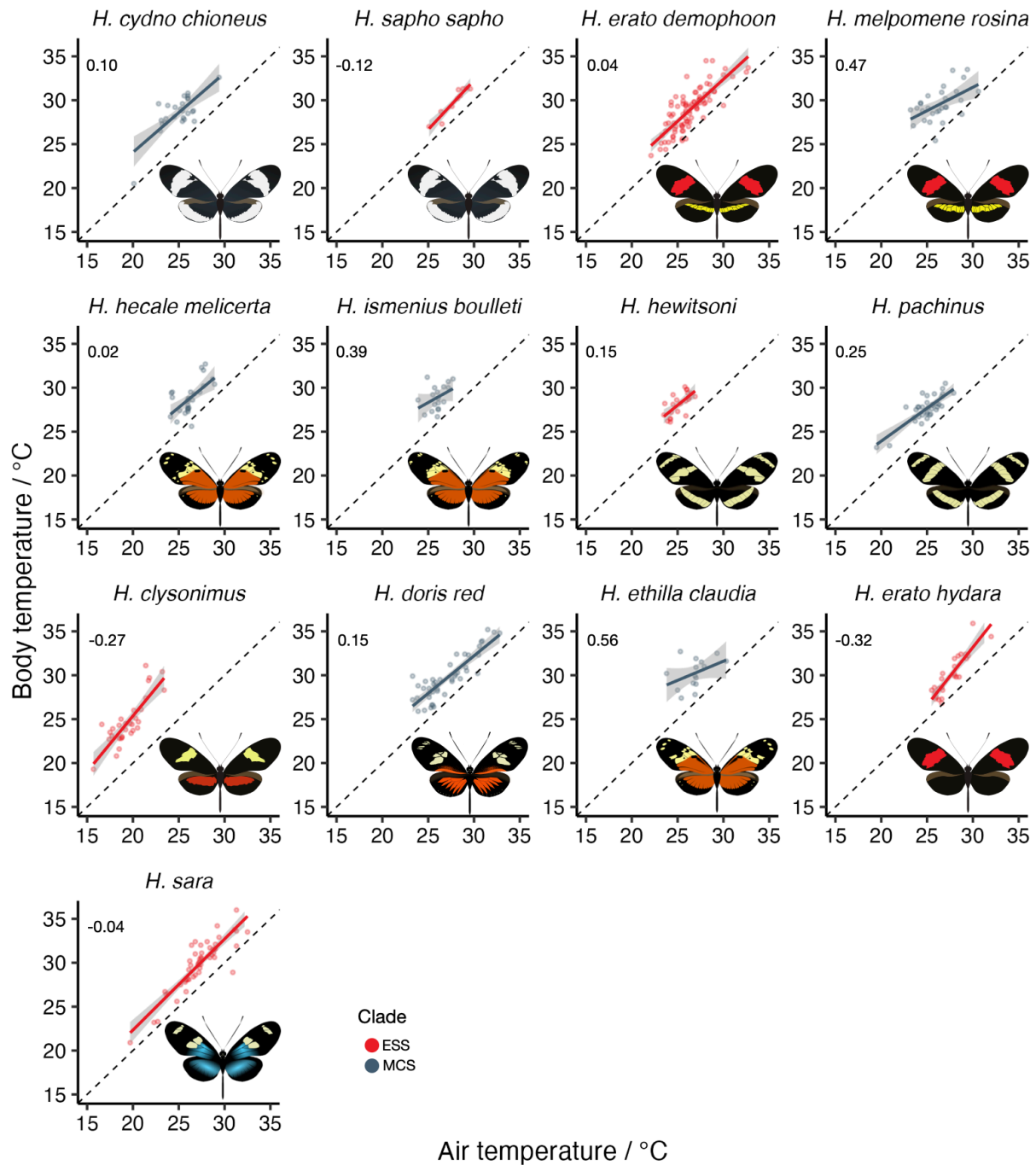
SUPPLEMENTARY FIGURE 4.4. Correlation among continuous predictor variables from the heating rate of live butterflies. Correlation between the percentage of black on the fore- and hindwings, a proxy for colour lightness, and mean forewing length (mm) is medium.



SUPPLEMENTARY FIGURE 4.5. Correlation among continuous predictor variables from the heating rate of dead butterflies. Correlation between the percentage of black on the fore- and hindwings, a proxy for colour lightness, and mean forewing length (mm) is medium.

SUPPLEMENTARY TABLE 4.2. *Heliconius* species, with their clade, habitat type, percentage black on the wings (a proxy for colour lightness), iridescence, mean forewing length (mm), mean wing load, pair number and buffering ability (calculated as the slope of the regression of thoracic temperature against air temperature, subtracted from 1, following Bladon et al 2020), take-off temperature (°C) and live and dead heating rate (°C/min–1) sample size (n), mean ($\bar{x}$) and standard error (SE).

Species	Clade	Habitat type	Black /%	Iridescence	Length/ mm	Wing load	Pair no.	Buffering ability estimate			Take-off temperature / °C			Live heating rate / °C/min–1			Dead heating rate / °C/min–1		
								n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE
<i>H. cydno chioneus</i>	MCS	Closed canopy forest	60	Y	40.157	0.182	1	26	0.10	0.16	16	34.06	1.05	10	12.14	0.65	6	16.30	0.51
<i>H. sapho sapho</i>	ESS	Closed canopy forest	60	Y	36.483	0.163	1	10	- 0.12	0.13	37	38.48	0.47	34	11.27	0.36	5	18.70	1.04
<i>H. erato demophoon</i>	ESS	Edge and gaps	70	N	33.562	0.168	2	90	0.04	0.08	32	35.66	0.76	21	13.75	0.52	5	15.80	0.60
<i>H. melpomene rosina</i>	MCS	Edge and gaps	70	N	36.135	0.177	2	28	0.47	0.16	47	35.32	0.49	24	13.10	0.46	5	16.40	1.07
<i>H. hecale melicerta</i>	MCS	Mixed	50	N	43.380	0.170	3	27	0.14	0.22	21	34.40	1.07	12	10.99	0.64	6	17.60	0.75
<i>H. ismenius bouletti</i>	MCS	Mixed	50	N	41.938	0.159	3	18	0.42	0.34	12	34.39	1.34	7	12.17	0.83	5	15.90	0.64
<i>H. hewitsoni</i>	ESS	Mixed	70	Y	37.177	0.168	4	22	0.15	0.20	10	33.01	1.17	-	-	-	4	18.20	0.44
<i>H. pachinus</i>	MCS	Mixed	70	Y	37.825	0.180	4	35	0.25	0.10	21	34.17	0.70	10	12.36	0.45	5	17.30	0.44
<i>H. erato etylus</i>	ESS	Edge and gaps	70	N	36.773	0.160	5	-	-	-	12	33.15	1.45	5	10.84	1.14	6	17.50	0.68
<i>H. melpomene ecuadorensis</i>	MCS	Edge and gaps	70	N	37.048	0.170	5	-	-	-	22	36.06	1.07	13	13.31	0.27	5	16.20	0.45
<i>H. erato notabilis</i>	ESS	Edge and gaps	90	N	37.125	0.166	6	-	-	-	20	38.37	0.90	14	12.65	0.56	6	17.70	0.61
<i>H. melpomene plesseni</i>	MCS	Edge and gaps	90	N	36.930	0.171	6	-	-	-	9	37.22	1.47	-	-	-	6	18.50	0.52
<i>H. erato lativitta</i>	ESS	Edge and gaps	50	N	35.778	0.155	7	-	-	-	9	37.18	1.69	6	14.48	0.86	-	-	-
<i>H. melpomene malletti</i>	MCS	Edge and gaps	80	N	37.920	0.179	7	-	-	-	36	36.80	0.49	28	12.61	0.39	6	19.30	0.24
<i>H. clysonimus</i>	ESS	High altitude	70	N	39.119	0.174	-	31	- 0.27	0.16	-	-	-	-	-	-	3	16.90	0.70
<i>H. himera</i>	ESS	High altitude	70	N	33.200	0.166	-	-	-	-	7	29.50	1.97	-	-	-	-	-	-
<i>H. doris</i>	MCS	Mixed	60	N	40.079	0.169	-	44	0.15	0.07	-	-	-	-	-	-	5	17.70	0.55
<i>H. erato hydara</i>	ESS	Edge and gaps	80	N	37.650	0.161	-	26	- 0.32	0.14	-	-	-	-	-	-	-	-	-
<i>H. ethilla claudia</i>	MCS	Closed canopy forest	50	N	39.510	0.175	-	15	0.56	0.26	-	-	-	-	-	-	-	-	-
<i>H. numata bicoloratus</i>	MCS	Closed canopy forest	60	N	41.533	0.158	-	-	-	-	18	34.61	0.84	9	14.10	0.78	-	-	-
<i>H. numata tarapotensis</i>	MCS	Closed canopy forest	50	N	40.724	0.159	-	-	-	-	17	32.78	1.06	-	-	-	-	-	-
<i>H. numata illustris</i>	MCS	Closed canopy forest	30	N	40.011	0.152	-	-	-	-	8	31.76	1.40	-	-	-	-	-	-
<i>H. sara</i>	ESS	Mixed	90	Y	33.827	0.166	-	52	- 0.04	0.08	-	-	-	-	-	-	6	18.40	0.24



SUPPLEMENTARY FIGURE 4.6. *Heliconius* body temperatures (°C) at different air temperatures (°C) for each subspecies by clade (*Erato*/*Sara*/*Sapho* (ESS) = red, *Melpomene* /*Cydno*/*Sylvaniform* (MCS) = blue). Solid lines represent the modelled relationship between body temperature and air temperature. The grey bands show 95% confidence intervals. The black dashed line represents a 1:1 relationship between body and air temperature. Buffering ability estimate is in the top left corner of each plot and is calculated as the slope of a simple linear regression, with body temperature as the response variable and air temperature as the predictor variable, subtracted from one. Butterfly illustration credit: Steven Van Belleghem.

SUPPLEMENTARY TABLE 4.3. *Model output for generalised linear mixed models of a. buffering ability (calculated as the slope of the regression of thoracic temperature against air temperature (T_{air}), subtracted from 1, following Bladon et al 2020), b. take-off temperature and c. live and d. dead heating rate. Bold indicates significant effects.*

a			
Random effects			
Groups	Variance	Std.Dev.	
Species	0.103	0.321	
Residual	1.831	1.353	
Fixed effects			
Term	Estimate	Std. Error	Pr(> t)
(Intercept)	7.399	6.580	0.262
T_{air}	0.356	0.211	0.093
Colour lightness	-2.141	0.797	0.008
Wing load	3.166	0.997	0.002
Wing aspect ratio	3.737	1.013	0.003
T_{air}:Colour lightness	0.077	0.030	0.010
b			
Random effects			
Groups	Variance	Std.Dev.	
Species	3.832	1.958	
Residual	15.700	3.962	
Fixed effects			
Term	Estimate	Std. Error	Pr(> t)
(Intercept)	34.959	0.519	<0.001
c			
Random effects			
Groups	Variance	Std.Dev.	
Species	0.393	0.627	
Residual	3.988	1.997	
Fixed effects			
Term	Estimate	Std. Error	Pr(> t)
(Intercept)	22.822	2.079	<0.001
Forewing length	-2.639	0.545	<0.001
Iridescence	-1.359	0.548	0.056
d			
Random effects			
Groups	Variance	Std.Dev.	
Species	0.587	0.766	
Residual	1.862	1.365	
Fixed effects			
Term	Estimate	Std. Error	Pr(> t)
(Intercept)	4.915	3.579	0.175
Forewing length	0.217	0.072	0.003
Colour lightness	0.623	0.214	0.010

SUPPLEMENTARY TABLE 4.4. *Model output for phylogenetic generalised linear mixed models (PGLMMs) of the effect of morphological traits on a. buffering ability, b. take-off temperature, c. live heating rate and d. dead heating rate. Bold indicates significant effects; coefficients where the 95% Bayesian credible interval (CI) does not overlap zero.*

a				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.008	<0.001	0.001
1 sp__	<0.001	0.015	<0.001	0.015
residual	1.887	1.374	1.654	2.171
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	21.434	10.184	32.681	
Tair	0.254	-0.152	0.661	
Colour lightness	-2.342	-3.882	-0.802	
Wing load	-0.842	-1.285	-0.400	
Wing aspect ratio	3.114	1.801	4.430	
Tair:Colour lightness	0.086	0.029	0.143	
b				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	2.827	1.681	1.197	7.708
1 sp__	<0.001	0.007	<0.001	<0.001
residual	15.610	3.951	13.490	18.298
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	34.981	34.507	35.455	
c				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.007	<0.001	0.001
1 sp__	<0.001	0.007	<0.001	0.001
residual	4.172	2.042	3.441	5.144
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	21.907	18.528	25.286	
Forewing.length	-2.410	-3.309	-1.512	
IridescenceY	-1.395	-2.042	-0.748	
d				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.007	<0.001	0.001
1 sp__	<0.001	0.007	<0.001	0.001
residual	2.319	1.523	1.743	3.202
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	5.543	-0.793	11.879	
Forewing.length	0.203	0.071	0.334	
Black	0.608	0.306	0.911	

SUPPLEMENTARY TABLE 4.5. *Heliconius* species, clade and pair number. For buffering ability estimate results of the likelihood ratio test between models with and without the two-way species:air temperature interaction, and air temperature are given (test-statistic (F), degrees of freedom (df) and likelihood p-value). For take-off temperature and heating rate of live and dead butterflies, results of the likelihood ratio test between models with and without species are given (test-statistic (F), degrees of freedom (df) and likelihood p-value (p)). Statistically significant p-values are in bold and indicate a significant difference in buffering ability, take-off temperate or heating rate between species within memetic pairs.

Species	Clade	Pair number	Buffering ability estimate			Take-off temperature / °C			Live heating rate / °C/min-1			Dead heating rate / °C/min-1		
			F	df	p	F	df	p	F	df	p	F	df	p
<i>H. sapho sapho</i>	ESS	1	0.423	1	0.520	20.121	1	<0.001	1.370	1	0.248	4.905	1	0.054
<i>H. cydno chioneus</i>	MCS													
<i>H. erato demophoon</i>	ESS	2	6.19	1	0.014	0.156	1	0.694	0.872	1	0.356	0.271	1	0.617
<i>H. melpomene rosina</i>	MCS													
<i>H. hecale melicerta</i>	ESS	3	0.447	1	0.508	0.000	1	0.996	1.249	1	0.279	2.876	1	0.124
<i>H. ismenius bouletti</i>	MCS													
<i>H. hewitsoni</i>	ESS	4	0.204	1	0.654	0.811	1	0.375	-	-	-	2.171	1	0.184
<i>H. pachinus</i>	MCS													
<i>H. erato etylus</i>	ESS	5	-	-	-	2.608	1	0.116	9.489	1	0.007	2.346	1	0.157
<i>H. melpomene ecuadorensis</i>	MCS													
<i>H. erato notabilis</i>	ESS	6	-	-	-	0.479	1	0.495	-	-	-	1.050	1	0.330
<i>H. melpomene plesseni</i>	MCS													
<i>H. erato lativitta</i>	ESS	7	-	-	-	0.091	1	0.765	4.010	1	0.054	-	-	-
<i>H. melpomene malletti</i>	MCS													

SUPPLEMENTARY TABLE 4.6. *Model output for phylogenetic generalised linear mixed models (PGLMMs) of the effect of habitat type on a. buffering ability, b. take-off temperature, c. live heating rate and d. dead heating rate. Bold indicates significant effects; coefficients where the 95% Bayesian credible interval (CI) does not overlap zero.*

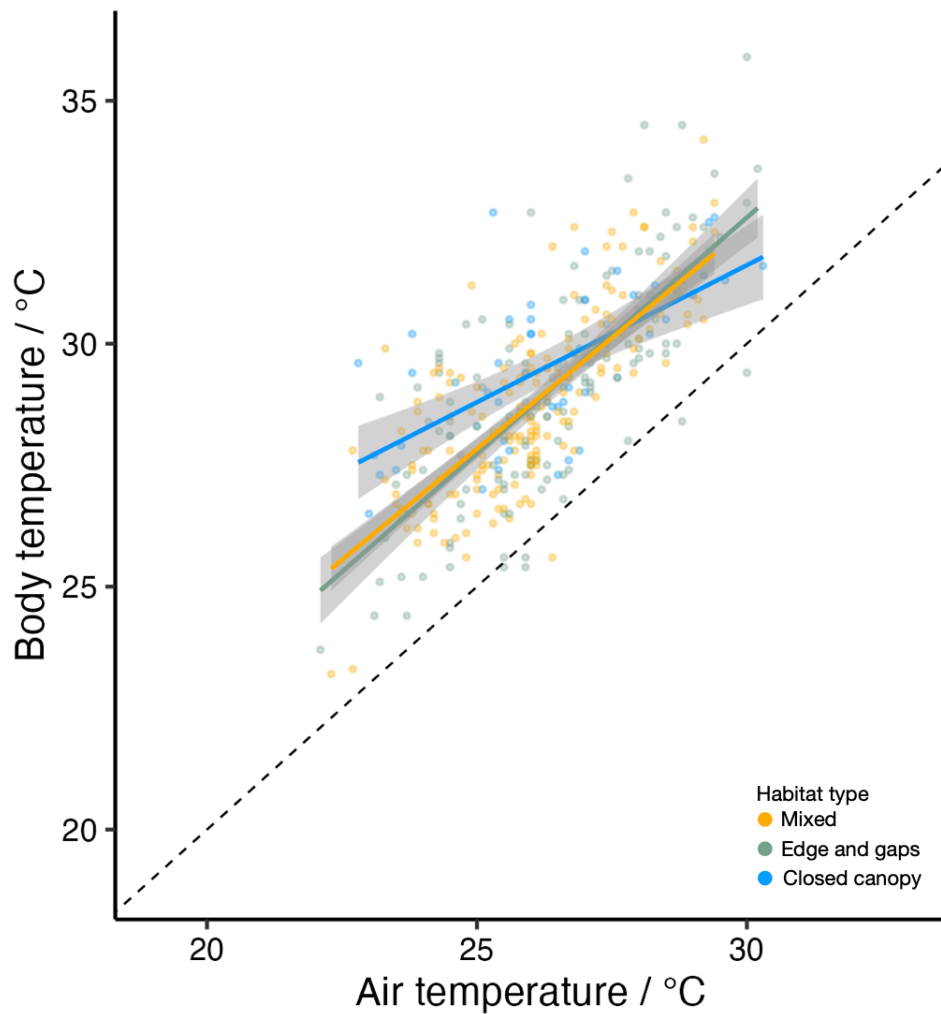
a				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	<0.001	<0.001	<0.001
1 sp__	<0.001	<0.001	<0.001	<0.001
residual	1.822	1.350	1.591	2.112
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	10.295	5.482	15.108	
Tair	0.728	0.545	0.912	
Habitat type Edge and gaps	-5.277	-10.946	0.393	
Habitat type Mixed	-5.247	-10.579	0.085	
Tair:Habitat type Edge and gaps	0.182	-0.033	0.398	
Tair:Habitat type Mixed	0.182	-0.022	0.385	
b				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	0.1716	0.4142	0.03359	2.513
1 sp__	0.154	0.3924	0.03137	2.188
residual	15.7824	3.9727	13.60367	18.524
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	35.098	33.583	36.662	
Habitat type Edge and gaps	0.982	-1.401	3.197	
Habitat type High altitude	-5.925	-10.324	-1.529	
Habitat type Mixed	-1.121	-3.367	1.116	
c				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.007	<0.001	0.001
1 sp__	<0.001	0.007	<0.001	0.001
residual	4.752	2.180	3.916	5.856
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	11.913	11.323	12.503	
Habitat type Edge and gaps	1.128	0.411	1.845	
Habitat type Mixed	-0.167	-1.158	0.825	
d				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.007	<0.001	0.001
1 sp__	<0.001	0.007	<0.001	0.001
residual	2.810	1.676	2.108	3.887
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	17.399	16.399	18.399	
Habitat type Edge and gaps	0.006	-1.124	1.135	
Habitat type High altitude	-0.499	-2.659	1.661	
Habitat type Mixed	0.139	-1.025	1.303	

SUPPLEMENTARY TABLE 4.7. *Mean values for buffering ability estimate (calculated as the slope of the regression of body temperature against air temperature, subtracted from 1, following Bladon et al 2020), take-off temperature and heating rate of live and dead butterflies and the corresponding standard errors (SE) and number of species (n) across habitat type.*

Habitat type	Buffering ability estimate			Take-off temperature / °C			Live heating rate / °C/min–1			Dead heating rate / °C/min–1		
	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE	n	$\bar{x}$	SE
Mixed	198	0.91	0.04	64	34.10	0.51	29	11.75	0.37	31	17.50	0.25
Edge and gaps	144	0.91	0.06	187	36.11	0.30	111	13.04	0.21	40	17.40	0.28
Closed canopy	51	0.28	0.10	96	35.45	0.44	53	11.91	0.32	11	17.40	0.64
High altitude	-	-	-	7	29.50	1.97	-	-	-	3	16.90	0.70

SUPPLEMENTARY TABLE 4.8. *Model output for phylogenetic generalised linear mixed models (PGLMMs) of the effect of habitat type on a. buffering ability where air temperature has been restricted to only include air temperatures within the range experienced in all habitat types and b. take-off temperature, where the one high altitude species, H. himera, was removed from the analysis. Bold indicates significant effects; coefficients where the 95% Bayesian credible interval (CI) does not overlap zero.*

a				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.006	<0.001	0.000
1 sp__	<0.001	0.007	<0.001	0.001
residual	1.725	1.313	1.500	2.009
Fixed effects				
Term	Estimate	lower.CI	upper.CI	
(Intercept)	14.531	9.347	19.714	
Tair	0.570	0.373	0.768	
Habitat type Edge and gaps	-11.063	-17.234	-4.889	
Habitat type Mixed	-9.511	-15.609	-3.409	
Tair:Habitat type Edge and gaps	0.401	0.166	0.635	
Tair:Habitat type Mixed	0.342	0.110	0.575	
b				
Random effects				
Term	Variance	Std.Dev	lower.CI	upper.CI
1 sp	<0.001	0.457	<0.001	1.900
1 sp__	0.215	0.464	0.050	2.041
residual	15.596	3.949	13.385	18.277
Fixed effect				
Term	Value	lower.CI	upper.CI	
(Intercept)	35.191	33.737	36.702	
Habitat type Edge and gaps	0.835	-1.597	3.081	
Habitat type Mixed	-1.273	-3.458	0.838	



SUPPLEMENTARY FIGURE 4.7. *Butterfly body temperatures (°C) at different air temperatures (°C) from different habitats: mixed (yellow), edge and gaps (green) and closed canopy (light blue). solid lines represent the modelled relationship between body temperature and air temperature, the grey bands show 95% confidence intervals, and the black dashed line represents a 1:1 relationship between body and air temperature. Air temperature was restricted to only include air temperatures within the range experienced in all habitat types.*

Chapter 5

Summary

In this thesis I have combined natural history, ecology and physiology to gain an insight into butterfly thermoregulation. I investigated how butterflies from different habitats and climates vary in their thermal vulnerability, the extent to which thermoregulation contains a phylogenetic signal, how morphology drives thermoregulatory abilities and the use of behavioural mechanisms in thermoregulation.

5.1 Thermoregulation across habitats and climates

In this thesis I have compared butterfly thermoregulation across neotropical and temperate regions (**Chapter 2**), bioclimatic regions in Europe (**Chapter 3**) and habitats (**Chapter 4**). Firstly, I compared neotropical and temperate butterfly buffering ability (ability to regulate body temperature relative to air temperature) and their use of microclimates and behaviour for thermoregulation (**Chapter 2**). Tropical species were thought to be more vulnerable to temperature changes than temperate species, however we lacked systematic field studies comparing the thermoregulatory ability of ectotherms across tropical and temperate regions (Deutsch et al., 2008; Johansson et al., 2020; Sunday et al., 2012). Contrary to a strong body of literature and my initial hypothesis, I found that at the assemblage level, neotropical butterflies were better at buffering than temperate butterflies. This was driven by neotropical butterflies (mainly Nymphalidae) lowering their temperature at high air temperatures, likely an adaptation to avoid high temperatures near their thermal limit. I have shown that neotropical butterflies are not likely to be more inherently vulnerable to warming than temperate species. This study is the first to my knowledge which directly compares thermoregulation of ectotherms across tropical and temperate regions using ecologically relevant trait measurements. Additionally, to my knowledge this is one of the first studies to measure thermoregulation in an assemblage of tropical butterflies, despite over 85% of the worlds insects residing in tropical regions (Bonebrake et al., 2010; Stork, 2018). Future work

should focus on furthering our understanding of the thermoregulatory abilities of tropical insects.

Secondly I studied the thermoregulatory ability of European butterfly populations distributed across regions and climates and investigated how they use microclimates and behaviour for thermoregulation. I further investigated the extent of regional differences in thermal traits and the likely drivers of this (temperature, precipitation, or geographic region) (**Chapter 3**). Studies have shown that some European butterflies are locally adapted to the specific climatic conditions they experience, with temperature and precipitation playing pivotal roles in shaping their population dynamics (Herrando et al., 2019; Melero et al., 2022). However, the mechanisms underpinning these results remains unknown and so I investigated this, looking at buffering ability and behavioural thermoregulation. In line with my hypothesis, I found that butterflies from warm regions were better at buffering than those from cold and cold temperate regions and this was driven by their ability to avoid heat stress at high air temperature. However, I found that they also heated up at low air temperatures, likely in an effort to remain active at cold temperatures. This is corroborated by my results from **Chapter 2**, which show that butterflies from a warmer neotropical region lower their body temperature at high air temperatures more so than butterflies from cooler temperate regions. I found intraspecific differences in buffering ability in a small number of species, which may be the result of local adaptation or acclimation to different aspects of the climate they experience. While temperature and precipitation are important drivers of butterfly thermoregulation, solar radiation and wind speed are also main determinants and future work should investigate if the patterns in buffering ability we show in part be driven by solar radiation or wing speed (Bonebrake et al., 2014).

Finally, I compared the thermal traits (buffering ability, take-off temperature and heating rate) of *Heliconius* butterflies from different habitats (mixed habitat, forest edge and gaps, closed canopy forest and high-altitude habitat). Studies have shown local adaptation and/or acclimation in butterfly thermal traits, however these studies are mostly focused on temperate butterflies or tropical species from two habitats (Dongmo et al., 2021; Kleckova et al., 2014; Montejo-Kovacevich et al., 2022). In line with my hypothesis, across habitats, local differences in thermoregulatory ability were observed, with *Heliconius* butterflies from closed

canopy forest habitats maintaining a more stable body temperature than butterflies from other habitats (**Chapter 4**). This was driven by their ability to raise their body temperatures when air temperatures were low, perhaps an adaptation to the cold forest interiors, enabling them to maintain activity at cold temperatures. The one high altitude *Heliconius* species that I studied had a lower take-off temperature than butterflies from low elevations. This may be an adaptation to the high solar radiation and/or low air temperatures experienced at high altitudes. By studying ecologically relevant thermal traits from a wide range of species my results build on earlier work, which found acclimation and adaptation to altitude in *Heliconius* species (Montejo-Kovacevich et al., 2020, 2022). My findings pave the way for reciprocal transplant experiments and genetic work which can determine if these traits are plastic, or if there are any genetic adaptations. Across these three studies (**Chapter 2-4**) I have shown that butterflies have unique thermoregulatory abilities as a result of the specific environment they inhabit. This might be a consequence of changes in morphology, behaviour, and/or physiology, traits which may also carry a phylogenetic signal.

5.2 Phylogeny

On order to determine if there is a phylogenetic signal in butterfly thermoregulation, the data in this thesis were analysed either in a phylogenetic framework (**Chapter 3-4**), or, where a species phylogeny was not available, were subset and analysed at the family level (**Chapter 2**). Additionally, I used the Müllerian mimicry exhibited in *Heliconius* butterflies to untangle the relative contributions of morphology and phylogeny in butterfly thermoregulation (**Chapter 4**). I compared thermoregulation in *Heliconius* mimetic pairs and grouped butterflies by their pair and compared thermoregulation between pairs. Any effect of relatedness in thermoregulatory ability would be indirect through a phylogenetic signal in thermal traits. When comparing the buffering ability of neotropical and temperate butterflies at the family level, only Nymphalidae and Riodinidae significantly differed in their buffering ability, with neotropical butterflies from each family being better at buffering than their temperate counterparts (**Chapter 2**). I could not make any assumptions about Riodinidae, as I only had one temperate species, however I could further explore the Nymphalidae data. I suggest that the better buffering ability of neotropical Nymphalidae may be due to their large size as they are bigger than temperate Nymphalidae and size is a determinant of buffering ability (Bladon

et al., 2020). When considering differences in buffering ability between butterfly populations within Europe and their use of microclimates and behaviour for thermoregulation, there was no phylogenetic signal in thermal traits (**Chapter 3**). This is unsurprising considering the high degree of intraspecific variation found. The same result was found in *Heliconius* butterflies, phylogeny did not account for differences in thermal traits between species (**Chapter 4**). To my knowledge this is the first study that utilises Müllerian mimics to test the effects of morphology and relatedness on species traits and paves the way for more work to use this evolutionary mosaic to tease apart the role of phylogenetic relatedness in functional traits. Previous studies have analysed butterfly thermoregulatory data within a phylogenetic framework (Wenda et al., 2021; Xing et al., 2016). Similarly to my results, they found a negligible phylogenetic effect on butterfly thermal traits (maximum and average heat gain) (Wenda et al., 2021). Another study analysed butterfly families separately, in order to determine if there were family-level differences in buffering ability and found that family explained most of the variation in buffering ability, likely as a result of colour differences between families (Bladon et al., 2020). I emphasise the importance of analysing butterfly thermoregulation data within a phylogenetic framework as the species traits which determine thermoregulatory ability, such as morphology and behaviour, might contain a phylogenetic signal.

5.3 Morphology

Considering morphology is a main driver of thermoregulation I investigated the relative contribution of forewing size, wing aspect ratio, colour lightness and iridescence to thermoregulatory ability. When comparing neotropical and temperate butterflies, I found that morphological traits were likely driving the differences in buffering ability that I observed between regions. Specifically, large butterflies and those with a high wing aspect ratio were better at buffering than small butterflies and those with a low wing aspect ratio (**Chapter 2**). In line with my hypothesis, I found that in *Heliconius* butterflies, morphology was more important than phylogeny in thermoregulatory ability, with light-coloured, iridescent and large butterflies cooling their body temperature more than dark-coloured, non-iridescent and small butterflies (**Chapter 4**). My results support the growing body of evidence showing that morphology is instrumental in butterfly thermoregulation and that large, light-coloured and

iridescent butterflies are able to thermoregulate more effectively than small, dark-coloured and non-iridescent butterflies (Bladon et al., 2020; Krishna et al., 2020; Shanks et al., 2015; Tsai et al., 2020; Xing et al., 2016). Additionally, I show for the first time, that wing aspect ratio drives butterfly thermoregulation. This finding underscores the importance of including wing aspect ratio when assessing the role of morphology in butterfly thermoregulation. I found that the morphological traits determining heating rate of dead and live butterflies differed, suggesting that behaviour and/or physiology also drives thermoregulation in *Heliconius* butterflies (**Chapter 4**).

5.4 Behaviour

In this thesis I investigated behavioural thermoregulation, including postural thermoregulation (positioning of the wings to reflect solar radiation) and microclimate selection (moving to a specific microclimate). When comparing neotropical and temperate butterflies, I found that temperate butterflies used postural thermoregulation to raise their body temperature more than neotropical butterflies (**Chapter 2**). This was probably an adaption to the cool climates they live in. Similarly, when comparing butterflies from different regions in Europe, butterflies from cold zones used postural thermoregulation to raise their body temperature more than those from cold temperate or warm zones (**Chapter 3**). Previous studies have investigated postural thermoregulation in butterflies and found that, as we have shown, it is an effective strategy to gain heat at low air temperatures (Kleckova & Klecka, 2016; Wenda et al., 2021). Studies have also shown that postural thermoregulation may diminish some of the direct negative effects of warming (Bonebrake et al., 2014; Kemp & Krockenberger, 2002; Tsai et al., 2020). Here, I show for the first time, how the use of this behaviour differs between neotropical and temperate species, and between European butterfly assemblages.

When comparing the use of microclimates for thermoregulation between neotropical and temperate butterflies, I found that there was no difference (**Chapter 2**). When investigating the use of microclimates for thermoregulation in European butterflies, I found that butterflies from Southern regions used microclimates more than butterflies from Central and North-western regions. With increasing air temperatures butterflies from dry and warm temperate

zones were more reliant on microclimates for thermoregulation than butterflies from other zones (**Chapter 3**). This result suggests that butterflies from dry and warm temperate zones are most at risk from rising temperatures due to climate change. My results build on the work of Kleckova and Klecka (2016), who found temperate butterflies from the *Erebia* genus used microclimates for thermoregulation. Microclimates may buffer some of the effects of warming, however the extent to which they help the less mobile life stages, including eggs, larvae and pupae, is unclear (Montejo-Kovacevich et al., 2020; Pincebourde & Woods, 2020). These life stages are thought to be more vulnerable to increasing temperatures than the adult stage, and so to fully understand how Lepidoptera will respond to warming we need to further our understanding of the thermoregulatory abilities of these less mobile life stages (Kingsolver et al., 2011). Additionally, changes in land use which alter the availability of different microclimates may impact species' capacity to cope with climate change (Jucker et al., 2020). This may result in population declines and extinction events, especially in tropical regions, where the rate of habitat degradation is higher than in other parts of the world (Achard et al., 2014; García-Robledo & Baer, 2021).

5.5 Concluding remarks

By combining natural history, ecological field studies and physiological laboratory experiments I have shown that butterfly thermoregulation is a multifaceted response to the thermal environment a species inhabits and is driven by morphology and behaviour. Determining the relative contributions of these morphological and behavioural traits is complex, requiring a multitude of ecologically relevant thermal trait measurements on live butterflies, and where applicable in the field. In part, I have laid the groundwork for future ecologists to investigate thermal traits of species across geographical and bioclimatic regions as well as untangle the various contributions of morphology and relatedness via utilising mimetic species. This thesis provides much needed research on the traits which may dictate species vulnerability to moderate warming with climate change, particularly of tropical species. Additionally, I highlight variation in thermoregulatory ability which makes some populations and species more vulnerable to warming, while others are more resilient. Furthering our knowledge in these fields will assist in understanding species vulnerability to climate and land-use change

and can inform management strategies which may mitigate some of the repercussions of warming and habitat degradation.

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Appendix A

Article: Thermoregulatory ability and mechanism do not differ consistently between neotropical and temperate butterflies

Chapter 2:

Laird-Hopkins, B. C., Ashe-Jepson, E., Basset, Y., Arizala Cobo, S., Eberhardt, L., Freiberga, I., Hellon, J., Hitchcock, G. E., Kleckova, I., Linke, D., Lamarre, G. P. A., McFarlane, A., Savage, A. F., Turner, E. C., Zamora, A. C., Sam, K., & Bladon, A. J. (2023). Thermoregulatory ability and mechanism do not differ consistently pattern neotropical and temperate butterflies. *Global Change Biology*, 00, 1-13. <https://doi.org/10.1111/gcb.16797>

BCLH and AJB should be considered joint first author.

RESEARCH ARTICLE

Thermoregulatory ability and mechanism do not differ consistently between neotropical and temperate butterflies

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Abstract

Climate change is a major threat to species worldwide, yet it remains uncertain whether tropical or temperate species are more vulnerable to changing temperatures. To further our understanding of this, we used a standardised field protocol to (1) study the buffering ability (ability to regulate body temperature relative to surrounding air temperature) of neotropical (Panama) and temperate (the United Kingdom, Czech Republic and Austria) butterflies at the assemblage and family level, (2) determine if any differences in buffering ability were driven by morphological characteristics and (3) used ecologically relevant temperature measurements to investigate how butterflies use microclimates and behaviour to thermoregulate. We hypothesised that temperate butterflies would be better at buffering than neotropical butterflies as temperate species naturally experience a wider range of temperatures than their tropical counterparts. Contrary to our hypothesis, at the assemblage level, neotropical species (especially Nymphalidae) were better at buffering than temperate species, driven primarily by neotropical individuals cooling themselves more at higher air temperatures. Morphology was the main driver of differences in buffering ability between neotropical and temperate species as opposed to the thermal environment butterflies experienced. Temperate butterflies used postural thermoregulation to raise their body temperature more than neotropical butterflies, probably as an adaptation to temperate climates, but the selection of microclimates did not differ between regions. Our findings demonstrate that butterfly species have unique thermoregulatory strategies driven by behaviour and morphology, and that neotropical species are not likely to be more inherently vulnerable to warming than temperate species.

Benita C. Laird-Hopkins and Esme Ashe-Jepson should be considered joint first author.

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behaviour, climate change, ecophysiology, ectotherms, insects, Lepidoptera, microclimate, tropics

1 | INTRODUCTION

Climate change is predicted to cause a rise in mean global temperatures, with larger increases at higher latitudes, as well as increases in the frequency of extreme temperature events (Lee et al., 2021). Temperature changes can cause shifts in physiology, morphology, life history and distribution of species (Angilletta, 2009; Franco et al., 2006; Parmesan, 2006; Sunday et al., 2014). Considering this, the effect of temperature change on species is and will be wide-reaching, with potential cascading effects to communities and ecosystems (Eggleton, 2020; Parmesan, 2006; Sunday et al., 2014). Consequently, there is an urgent need to understand how species respond to changing temperatures.

To understand and predict the impacts of climate change on communities, it is important to identify the traits that affect species' sensitivity to temperature (Diamond et al., 2011; Diamond & Yilmaz, 2018). These include morphological traits, such as size or colouration, as well as physiological and behavioural traits, and may be a consequence of the thermal environment a species inhabits (Bonebrake et al., 2014; González-Tokman et al., 2020; Stella et al., 2018; Wenda et al., 2021). On a global scale, a major difference between thermal environments is found between temperate and tropical regions, with a narrower temperature range and higher mean annual temperature in tropical regions than temperate regions (Lee et al., 2021). Janzen (1967) predicted that the lower temperature variation found in tropical compared to temperate regions may select for narrower thermal tolerances among tropical species (the 'seasonality' hypothesis). Thus, although the degree of warming is predicted to be greater at higher latitudes, tropical species may be more at risk to temperature changes than temperate species, as they are less able to cope with variability (Lee et al., 2021). In addition, tropical species live closer to their thermal maximum than temperate species, and therefore may be more vulnerable to the same level of warming (Deutsch et al., 2008; Huey et al., 2009; Sunday et al., 2019). As a result, small variations in temperature would have severe consequences for the survival and fitness of tropical species (Deutsch et al., 2008; Ghalambor, 2006). However, other studies have shown that some temperate species may be equally vulnerable to climate change (Colado et al., 2022; Johansson et al., 2020; Mi et al., 2022). For example, temperate species with short activity periods may only experience narrow ranges in temperature (Johansson et al., 2020) and may be selected for low thermal tolerance in a similar way to tropical species (Colado et al., 2022). Despite more than 50% of insects species being found in the tropics, there is a lack of systematic field studies investigating the thermoregulatory capabilities of tropical ectotherms, and comparing tropical and temperate species (Deutsch et al., 2008; Johansson et al., 2020; Stork, 2018; Sunday et al., 2012). It remains unclear the extent to

which species from different ecological backgrounds and latitudes differ in their sensitivity to climate change.

The impact of climate change on species is often tested at the macroclimate scale, using coarse-scale weather station data, assuming that species live at these ambient temperatures (Diamond & Yilmaz, 2018; Pincebourde & Woods, 2020). This does not take into consideration the small-scale microclimate differences in temperature that individual species experience in heterogeneous environments, nor the fact that for small ectotherms moving just a small distance in these environments can result in exposure to very different climatic conditions (Pincebourde & Woods, 2020). Indeed, temperatures between microclimates and weather stations can differ significantly, with weather stations overestimating annual mean temperature in tropical understory forest by up to 0.5°C and in highlands by up to 2.0°C (Montejo-Kovacevich et al., 2020). Small-scale temperature variation can play an important role in species' thermal adaptation, and can both buffer or magnify the effects of increasing temperatures (Pincebourde & Woods, 2020). We lack knowledge of how rising temperatures at the macroclimate scale impact microclimate temperatures, and how species are affected by these fine scale differences in temperature.

Butterflies are important as an indicator taxa, pollinators, prey and herbivores, but as ectotherms may be particularly vulnerable to temperature change (Bonebrake et al., 2010; Diamond & Yilmaz, 2018; Ma et al., 2021; Menéndez et al., 2007). Understanding how they respond to temperature change is crucial for assessing the ecosystem-wide impacts of climate change (Harvey et al., 2020). Butterflies can regulate their body temperature in a variety of ways, including by selecting favourable thermal microclimates during different life stages or at different times of day (Cómbita et al., 2022; De Frenne et al., 2021; Kearney et al., 2009; Kleckova et al., 2014). They also use other behavioural mechanisms, such as the orientation of their wings relative to the sun, to increase or reduce thermal absorbance (Kemp & Krockenberger, 2002; Shanks et al., 2015). Morphological characteristics, including wing colour, reflectance, size and hair length, can also be important in determining thermoregulatory ability (Bonebrake et al., 2014; Stella et al., 2018; Wenda et al., 2021). For example, light-coloured butterflies can regulate their body temperature by using their wings to reflect solar radiation onto their thorax to heat up, or back into the environment to cool down (Shanks et al., 2015; Zeuss et al., 2014). Dark-coloured butterflies can heat up quicker than light-coloured butterflies, perhaps explaining why they are found more frequently in cooler climates, such as forest interiors or higher latitudes, where being able to heat up quickly increases fitness (Günter et al., 2019; Xing et al., 2016). Size can also determine thermoregulatory ability, with the thermoregulation of sympatric mountain butterflies differing between species

in relation to their habitat use and body size (Kleckova et al., 2014). Large species can use the surface area of their wings to intercept sunlight and are generally better at thermoregulating than small species (Bladon et al., 2020; Wenda et al., 2021). Finally, thermoregulatory ability differs systematically between butterfly families, with temperate Pieridae species being particularly good at thermoregulating compared to other temperate families (Bladon et al., 2020).

In this study, we compared buffering ability—the ability to regulate body temperature independently of the surrounding air temperature—of butterfly populations from neotropical (Panama) and temperate regions (Czech Republic, Austria and the United Kingdom). We hypothesised (H1) that butterflies from temperate regions, being naturally exposed to a greater range of temperatures, will be better able to buffer their body temperature against changes in air temperature than those from the neotropical region. We tested this at the assemblage and family level. Since there was significant overlap in butterfly species found at our European sites, and at our Panamanian sites, we treated them as single assemblages, resulting in a temperate group and a neotropical group of the most common and conspicuous butterflies in each region. If differences in buffering ability between neotropical and temperate regions were observed we hypothesised (H2) that this will be driven by the temperatures butterflies are exposed to in the different regions, not by differences in their morphological traits. Finally, we hypothesised (H3) that as an adaptation to temperate climates, temperate butterflies will actively select warmer microclimates than neotropical butterflies, and that they will heat up through postural means more than neotropical species.

2 | METHODS

2.1 | Study sites

Neotropical data were collected in Panama from February to June 2020 and from October 2021 to March 2022 during both wet (May–December) and dry (January–April) seasons (Figure S1; Table S1) (Leigh, 1999). Temperate data were collected in the Czech Republic and Austria between April and August 2021 and in the United Kingdom between April and September 2009 and May and September 2018 (Figure S1; Table S1) (Bladon et al., 2020). Data collection took place between 7:30 and 17:30. Neotropical field sites included lowland scrub and managed urban green spaces, secondary semi-deciduous lowland tropical forest, mountain rainforest and management agroforestry (Table S1). Temperate field sites included calcareous meadows, grassland meadows, alpine/montane grassland, encroaching scrub, secondary forest and exposed ground (Table S1).

2.2 | Butterfly body temperature and morphological measurements

Butterflies were captured with butterfly nets when encountered (without chasing) and data were collected following the protocol

used by Bladon et al. (2020), as follows. Once in the net, and within 10s, a temperature reading of the butterfly's thorax (body temperature, T_b) was taken using a thermocouple (0.5 mm diameter) and handheld indicator (Tecpel Thermometer 305B, TC Direct). Air temperature (T_a) was taken at waist height where the butterfly was caught, with the thermocouple shaded from the sun. If the butterfly was resting on a substrate before capture, the temperature of the air 1 cm above where it was sat was recorded with the thermocouple (microclimate temperature, T_m). The butterfly was identified to species or subspecies. In the case of butterflies from the tropical *Calephelis* genus it was not possible to identify individuals to species, so data from these butterflies were aggregated to genus level. Forewing length (in mm) from the tip of the wing to the point where it meets the thorax was measured using callipers (at the Panama and UK sites only).

We calculated mean forewing length from our field data for each of the Panama and UK species. With the exception of *Erebia* spp., for which there was field data (Laird-Hopkins, unpublished data), mean forewing lengths of butterflies from the Czech Republic and Austria were taken from the literature (Lindsey, 2016).

Wing aspect ratio (the ratio of forewing length: forewing width) strongly influences flight performance, which in turn can determine a species' capacity to move to more favourable microclimates and hence their ability to buffer air temperature (Chazot et al., 2016). Wing aspect ratio of all neotropical and temperate species was calculated from photographs sourced from the literature (Table S2). To do this, photographs of five female and five male mounted specimens of each species were sourced and uploaded into ImageJ (Schneider et al., 2012). The forewing length (as above) and depth (longest line between the leading and trailing edges of the forewing, measured perpendicular to the wing length axis) were measured, and used to calculate forewing aspect ratio for each specimen. Mean aspect ratio was then calculated for each species. For sexually dimorphic species, the mean forewing aspect ratio of females and males was calculated separately.

Colour scale was determined for each species based on the lightness/darkness of their wings, following Bladon et al. (2020) (ranked: 1—white, 2—yellow-green, 3—orange, 4—orange-brown/blue, 5—brown and 6—black). If the difference in colour between males and females was sufficient to classify them in different categories, the mean colour category of females and males was used.

2.3 | Statistical analysis

2.3.1 | Preliminary analysis

To ensure our estimates of buffering ability were robust, only species with at least 10 T_b measurements across a range of at least 5°C of T_a were included in analyses. To estimate the buffering ability of each species, a simple linear regression with T_b as the response variable and T_a as the predictor variable was fitted separately for each species. The slope of the relationship was extracted and, following Bladon et al. (2020), subtracted from one to provide an estimate of

buffering ability (buffering ability estimate). With this method, a steep slope shows that the butterfly's T_b varies greatly over small ranges of T_a , thus indicating a low buffering ability. A shallow slope shows that the butterfly's T_b remains relatively stable over wide ranges of T_a , thus indicating a high buffering ability.

2.3.2 | Do temperate and neotropical butterfly assemblages and families differ in buffering ability?

In total 94 species, across six families (Hesperiidae, Lycaenidae, Nymphalidae, Papilionidae, Pieridae and Riodinidae) were sampled in our study regions (neotropical: 54 species, $n=1333$; temperate: 40 species, $n=5482$). There is only one Riodinidae species (*Hamearis lucina*) found in Europe, so our analysis for this family specifically compared this species' buffering ability to those of its neotropical relatives. Papilionidae species were only sampled in the neotropical region, so this family was removed when comparing the buffering ability within families across neotropical and temperate regions.

To test for differences in buffering ability between butterfly assemblages, and within each butterfly family, in neotropical and temperate regions, linear mixed effects models were fitted for the assemblage and for each family separately. Body temperature was fitted as the response variable and T_a , region (temperate or neotropical) and their two-way interaction as predictor variables. Species was included as a random effect. A likelihood ratio test was performed to determine if the model with the two-way interaction was a better fit than the model without the term; if so, it would suggest that butterfly assemblages, or families, in the two regions differ in their buffering ability. The slope estimates for each region (neotropical and temperate) were extracted and subtracted from one as described above, to obtain a mean buffering ability estimate across each assemblage and family in each region.

2.3.3 | When controlling for morphology, do temperate and neotropical butterflies differ in buffering ability?

Morphological characteristics are known to be important in determining species' thermoregulatory abilities (Bladon et al., 2020; Shanks et al., 2015; Sheldon & Tewksbury, 2014; Xing et al., 2016). We first determined if morphological characteristics (mean forewing length, mean forewing aspect ratio and wing colour) differed between neotropical and temperate species. We fitted one-way ANOVAs with mean forewing length (mm), mean wing aspect ratio and wing colour separately as the response variable and region (neotropical or temperate) as the predictor variable.

To determine if forewing length, forewing aspect ratio and/or wing colour were driving differences in buffering ability between neotropical and temperate butterflies we fitted linear mixed effects models. Body temperature (T_b) was the response variable. Air temperature (T_a), region (temperate or neotropical), mean

forewing length, mean forewing aspect ratio and wing colour, and the two-way interactions between T_a and each of the other terms were predictor variables. Mean forewing length was $\log_{10}$ -transformed to ensure all predictor variables were on a similar scale. Species was included as a random effect. If the interaction between T_a and region was retained in the best-fitting model, it would suggest that buffering ability differs between regional assemblages in addition to any effect of morphology. To find the best-fitting model we used backward stepwise selection with the step function in R package 'lmerTest' (Kuznetsova et al., 2017). A preliminary Pearson correlation coefficient analysis indicated no correlation between the three continuous predictor variables (mean forewing length, mean forewing aspect ratio and wing colour), justifying their inclusion as predictor variables in the same models (Panel S1). Preliminary analysis fitting one-way ANOVAs with mean forewing length (mm), mean wing aspect ratio and wing colour separately as the response variable and family as the predictor variable showed that there was a significant effect of family on the three response variables (Table S3). Family was therefore not included in the linear mixed effects model described above.

2.3.4 | Do temperate and neotropical butterfly assemblages and families differ in their use of microclimate selection and postural thermoregulation, or in their index of thermal specialisation?

The data were subset so that only species with at least 10 microclimate temperature measurements (T_m) were included in this analysis. For the assemblage-level analysis, this included 39 species, 13 neotropical ($n=176$; families: five Hesperiidae, seven Nymphalidae and one Riodinidae) and 26 temperate ($n=919$; families: three Hesperiidae, four Lycaenidae, 15 Nymphalidae and four Pieridae) (Table S4). For the family-level analysis there were only sufficient data from both temperate and neotropical regions for Hesperiidae and Nymphalidae to undertake this analysis. This included five neotropical and three temperate Hesperiidae (neotropical: $n=57$; temperate: $n=56$), and seven neotropical and 15 temperate Nymphalidae (neotropical: $n=101$; temperate: $n=499$).

For each individual butterfly, we calculated the extent to which its chosen T_m differed from the surrounding T_a , by subtracting T_a from T_m ('microclimate selection', following Bladon et al. (2020)). A large difference between T_m and T_a indicates that the butterfly is selecting a specific thermal microclimate distinct from T_a . For each butterfly, we also calculated the extent to which T_b differed from the temperature of its chosen T_m , by subtracting T_m from T_b ('postural thermoregulation', following Bladon et al. (2020)). A large difference between T_m and T_b suggests that the butterfly is regulating its temperature independently of its chosen microclimate, for example by behavioural posturing relative to the sun. To quantify the relative contribution of microclimate selection and postural thermoregulation to each

butterfly's body temperature, we calculated an index of thermal specialisation (ITS) by subtracting microclimate selection ($T_m - T_a$) from postural thermoregulation ($T_b - T_m$). A large ITS value indicates that the butterfly is utilising postural thermoregulation more, and is able to alter its body temperature beyond that of its immediate environment, making it a thermal generalist. A small ITS value means that the butterfly relies more on microclimate selection for thermoregulation, and is a thermal specialist (Bladon et al., 2020).

To test whether the use of microclimate selection and postural thermoregulation, and the value of ITS, differed between the butterfly assemblage and within butterfly families from neotropical and temperate regions, linear mixed effects models were fitted for the assemblage, and each family separately. Microclimate selection, postural thermoregulation and ITS were fitted separately as response variables, and region (temperate or neotropical), T_a and their two-way interaction were included as predictor variables. Species was included as a random effect. A likelihood ratio test was performed to determine if the full model was a better fit for the data than a model without the interaction term between region and T_a ; if so, it would suggest that butterflies from different regions differed in their use of microclimate selection, postural thermoregulation or ITS across a range of T_a . An additional likelihood ratio test was performed to determine if the model including region and T_a as a predictor variable was a better fit than the model including only T_a ; if so, it would suggest that butterflies from different regions differed in their use of microclimate selection, postural thermoregulation or ITS, but independently of T_a .

To control for differences in T_a between neotropical and temperate regions and to assess the robustness of our results, all analyses (1, 2 and 3) were repeated on a subset of data that were restricted to the same T_a range in both neotropical and temperate regions. All statistical analyses were undertaken in R version 4.1.2 (R Core Team, 2019). Graphics were produced using the package 'ggplot2' (Wickham, 2016). Linear mixed effects models were fitted using the 'lme4' package (Bates et al., 2015). To ensure normality and homogeneity in the residuals, graphical model diagnostics were applied to each model. The response parameter was $\log_{10}$ -transformed where necessary to achieve normality. Marginal means for neotropical and temperate species and families were predicted using the package 'ggeffects' (Lüdtke, 2018). The marginal effects of region, that is, the difference between marginal means, were used as a measure of effect size at the response scale.

3 | RESULTS

From the neotropical region, 1333 individuals from 54 species were measured. Air temperature (T_a) ranged from 17.4°C to 39.7°C, with a mean of 28.5°C. Body temperature (T_b) ranged from 22.1°C to 40.3°C, with a mean of 31.7°C. From the temperate region, 5482 individuals of 40 species were measured (Results S1). T_a ranged from 10.0°C to 34.8°C, with a mean of 22.2°C. T_b ranged from 15.4°C to 45.0°C, with a mean of 28.0°C. The buffering ability estimates for neotropical species ranged from -0.317 (*Hemiargus hanno*) to 1.005

(*Phoebis argante*), with a mean of 0.295 (Figure S2; Tables S4 and S5). The buffering ability estimates for temperate species ranged from -0.404 (*Hamearis lucina*) to 0.675 (*Erebia medusa*), with a mean of 0.243 (Figure S2; Table S4).

3.1 | Do temperate and neotropical butterfly assemblages differ in buffering ability?

Across the whole assemblage, the mean buffering ability of neotropical butterflies (0.350 ± 0.035) was significantly higher than the buffering ability of temperate butterflies (0.220 ± 0.010), with neotropical species appearing better able to reduce their T_b at higher T_a ($\chi^2 = 14.17$, $df = 1$, $p < .001$; Figure 1). This means that across a 20.0°C range of T_a , predicted neotropical butterfly T_b would vary by 13.0°C, while predicted temperate butterfly T_b would vary by 15.6°C. A similar result was obtained when data were restricted to the range of T_a which occurred in both regions (Figure S3; Results S2).

3.2 | Do temperate and neotropical butterfly families differ in buffering ability?

In the neotropical region, the buffering ability of butterfly families ranged from -0.020 (Lycaenidae) to 0.426 (Pieridae). This means that

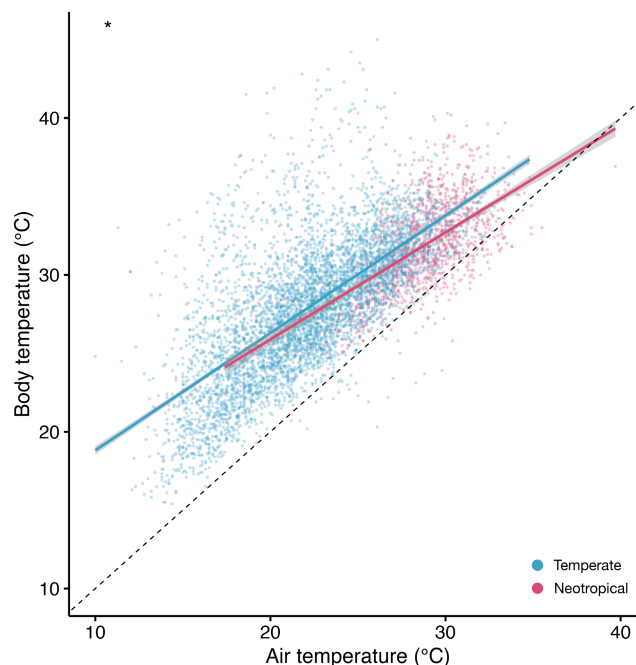


FIGURE 1 Individual butterfly body temperatures (°C) at different air temperatures (°C) from neotropical (pink) and temperate (blue) regions. Solid lines represent the modelled relationship between body temperature and air temperature. The grey bands show 95% confidence intervals. The black dashed line represents a 1:1 relationship between body and air temperature. The asterisk marks the significant difference in buffering ability estimate between neotropical and temperate butterflies.

across a 20.0°C range of T_a , predicted neotropical Lycaenidae T_b would vary by 20.4°C, while predicted neotropical Pieridae T_b would vary by 17.2°C. In the temperate region, family-level buffering ability ranged from -0.404 (Riodinidae) to 0.326 (Pieridae) (Table S6). This means that across a 20.0°C range of T_a , predicted temperate Riodinidae T_b would vary by 28.1°C, while predicted temperate Pieridae T_b would vary by 20.2°C. Neotropical Nymphalidae were better at buffering their T_b against changes in T_a than Nymphalidae from temperate regions ($\chi^2=21.66$, $df=1$, $p<.001$), and Riodinidae from neotropical regions were better at buffering their T_b against changes in T_a than *Hamearis lucina*, the only temperate Riodinidae species ($\chi^2=11.83$, $df=1$, $p<.001$). This was driven by neotropical Nymphalidae and Riodinidae being better able to lower their T_b at high T_a compared to temperate Nymphalidae and Riodinidae (Figure 2). This means that across a 20°C range of T_a , predicted temperate and neotropical Nymphalidae T_b would vary by 16.4 and 12.1°C, respectively, and predicted temperate and neotropical Riodinidae T_b would vary by 28.1 and 12.5°C respectively. There were no differences in family-level buffering ability between temperate and neotropical species for the other three families (Figure 2; Table S6).

When data were restricted to the range of air temperatures which occurred in both regions, a similar result was obtained for Nymphalidae and Pieridae, while Hesperidae and Lycaenidae showed better buffering ability in temperate than neotropical regions (Figure S4; Table S7). Specifically, temperate Hesperidae and Lycaenidae increased their T_b at low T_a more so than neotropical Hesperidae and Lycaenidae. It was not possible to model the restricted dataset for Riodinidae, due to limited temperature overlap between regions for the dataset (Results S3).

3.3 | When controlling for morphology do temperate and neotropical butterflies differ in buffering ability?

Mean forewing length was significantly larger in neotropical butterflies (26.7 ± 0.3 mm) than in temperate butterflies (19.9 ± 0.1 mm) ($F=581.75$, $df=1$, $p<.001$). Neotropical butterflies had a significantly higher mean wing aspect ratio (1.78 ± 0.01) than butterflies from temperate regions (1.71 ± 0.00) ($F=233.85$, $df=1$, $p<.001$).

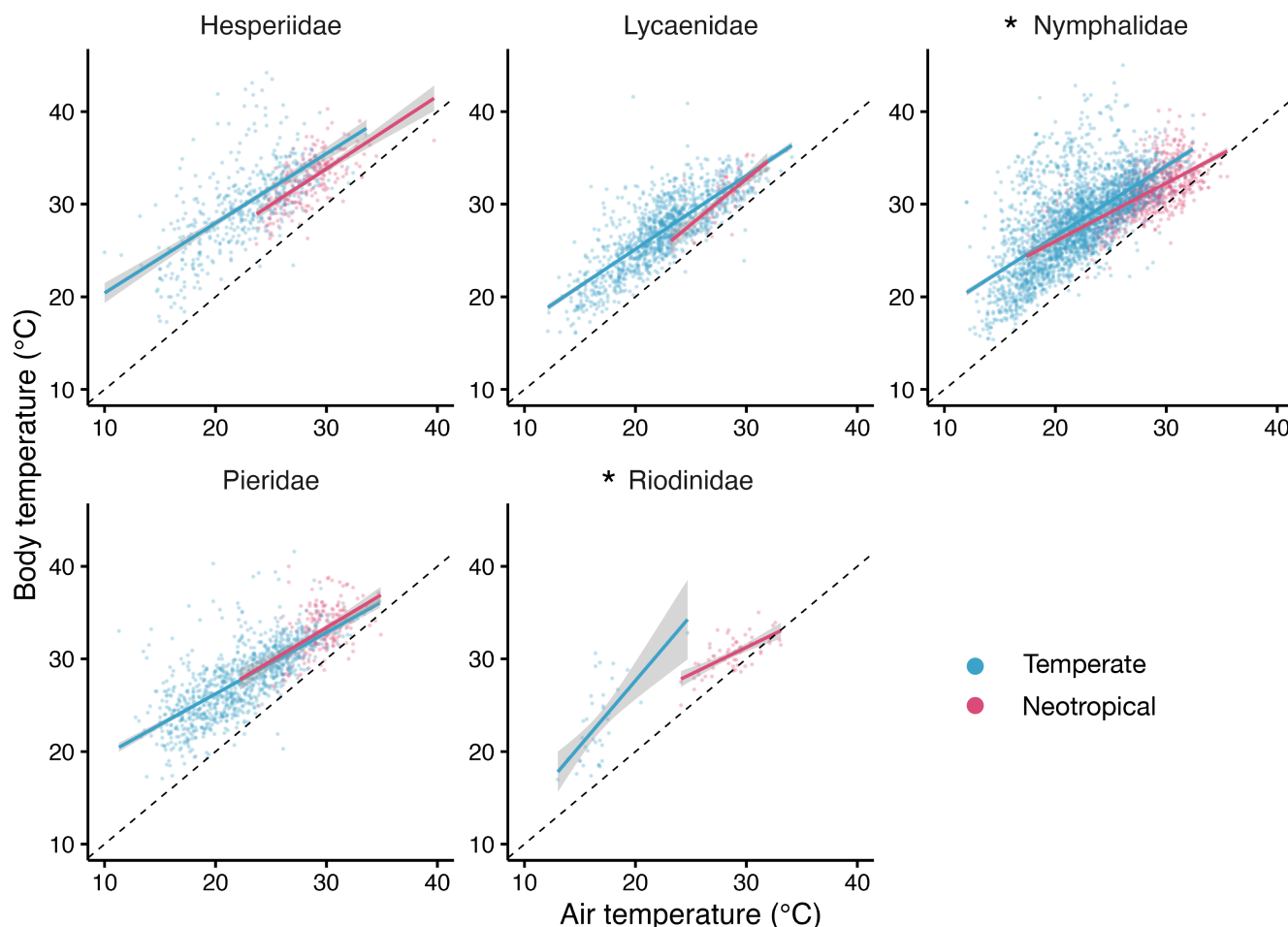


FIGURE 2 Individual butterfly body temperatures (°C) at different air temperatures (°C) from neotropical (pink) and temperate (blue) regions for each taxonomic family. Solid lines represent the modelled relationship between body temperature and air temperature. The grey bands show the 95% confidence intervals. The black dashed line represents a 1:1 relationship between body and air temperature. Families with a significant difference in buffering ability estimate between neotropical and temperate butterflies are marked with an asterisk.

Butterflies from neotropical regions were significantly darker (mean colour scale = 4.12 ± 0.04) than temperate butterflies (mean colour scale = 3.55 ± 0.02) ($F = 229.43$, $df = 1$, $p < .001$). The best-fitting model included T_a , region (temperate or neotropical), mean forewing length and mean forewing aspect ratio, and the two-way interactions between T_a and each of mean forewing length and mean forewing aspect ratio. This showed that there was no difference in buffering ability between neotropical and temperate butterflies when accounting for differences in morphology (Table 1). Differences in mean forewing length and mean forewing aspect ratio were the main drivers of differences in buffering ability between neotropical and temperate butterflies (Table 1). Butterflies with larger mean forewing lengths and higher mean wing aspect ratios (longer, shallower wings) were better at buffering than their T_b against changes in T_a than those with smaller mean forewing lengths and lower mean wing aspect ratios (shorter, deeper wings) (Table 1). The same result was found when the data were restricted to the range of air temperatures which occurred in both regions (Table S8).

3.4 | Do temperate and neotropical butterfly assemblages and families differ in their use of microclimate selection and postural thermoregulation, or in their index of thermal specialisation?

This analysis included a total of 26 temperate ($n = 919$) and 13 neotropical ($n = 176$) species. The use of microclimate selection was not significantly different between temperate and neotropical species ($\chi^2 = 0.104$, $df = 1$, $p = .747$) (Figure 3a, Table S9). This result was the same for the two families tested individually (Hesperiidae: $\chi^2 = 0.025$,

TABLE 1 Output from the best-fitting linear mixed effects model, with body temperature (T_b) as the predictor variable and air temperature (T_a), region (neotropical and temperate), mean forewing length, mean forewing aspect ratio and the two-way interaction between T_a and each of mean forewing length and mean forewing aspect ratio as response variables.

	Estimate	SE	df	F	p
Region: temperate	-20.196	4.590	1	22.137	<.001*
Region: tropical	-21.662	0.312			
T_a	1.792	0.156	1	132.181	<.001*
Mean forewing length	6.040	0.903	1	44.770	<.001*
Mean forewing aspect ratio	7.728	2.247	1	11.827	<.001*
T_a : Mean forewing length	-0.456	0.070	1	42.623	<.001*
T_a : Mean forewing aspect ratio	-0.252	0.763	1	10.947	<.001*

Note: Mean forewing length was $\log_{10}$ -transformed to ensure it was on the same scale as other predictor variables. Species was included as a random effect. Significant terms are marked with an asterisk.

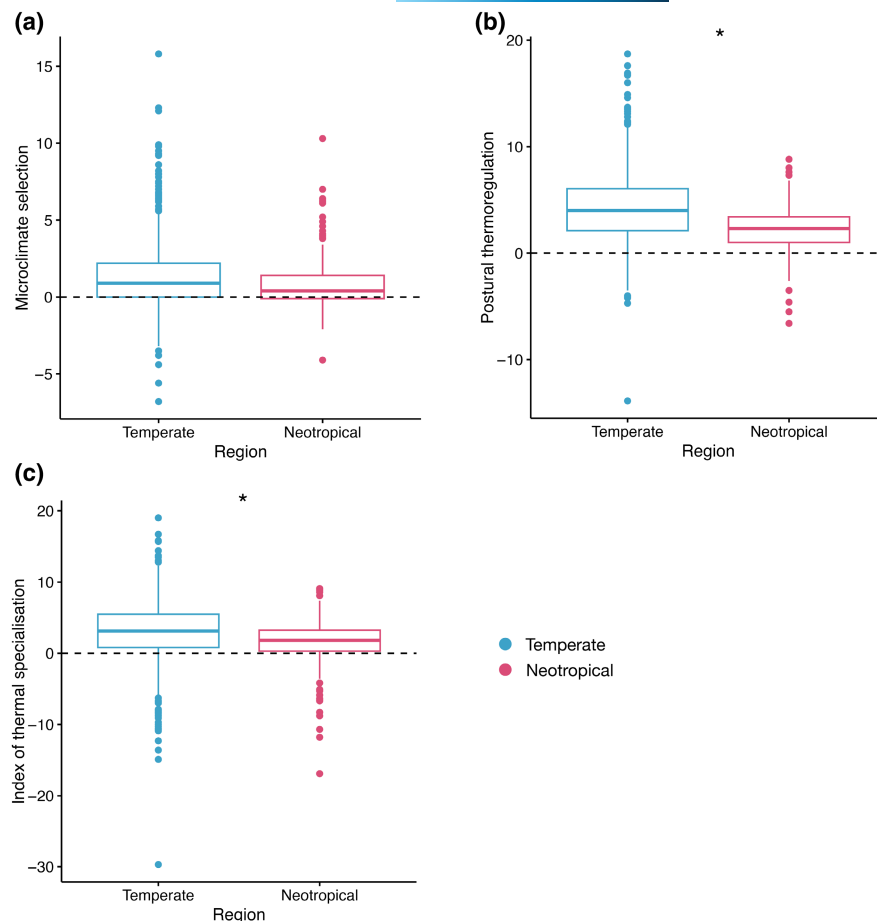
$df = 1$, $p = .875$; Nymphalidae: $\chi^2 = 0.007$, $df = 1$, $p = .932$). The use of postural thermoregulation was greater in temperate species than in neotropical ($\chi^2 = 8.314$, $df = 1$, $p = .004$), however, when testing families individually only Nymphalidae reflected this result ($\chi^2 = 11.86$, $df = 1$, $p < .001$); there was no difference between neotropical and temperate Hesperiidae ($\chi^2 = 2.431$, $df = 1$, $p = .119$) (Figure 3b; Table S9). Temperate species used postural thermoregulation to increase their body temperature by a mean of $4.2 \pm 0.1^\circ\text{C}$ compared to $2.2 \pm 0.2^\circ\text{C}$ in neotropical species. Temperate Nymphalidae used postural thermoregulation to increase their body temperature by a mean of $4.7 \pm 0.2^\circ\text{C}$ compared to $2.1 \pm 0.2^\circ\text{C}$ in neotropical Nymphalidae. The index of thermal specialisation (ITS) was greater in temperate than neotropical species ($\chi^2 = 7.150$, $df = 1$, $p = .008$) and when testing families individually the same was found in Nymphalidae ($\chi^2 = 7.970$, $df = 1$, $p = .005$), but not Hesperiidae ($\chi^2 = 2.516$, $df = 1$, $p = .113$) (Figure 3c; Table S9). Temperate species ITS was $2.9 \pm 0.1^\circ\text{C}$ compared to $1.4 \pm 0.3^\circ\text{C}$ in neotropical species and temperate Nymphalidae ITS was $3.3 \pm 0.2^\circ\text{C}$ compared to $1.2 \pm 0.3^\circ\text{C}$ in neotropical Nymphalidae. There was no effect of air temperature on the difference in how butterflies from neotropical and temperate regions used microclimate selection, postural thermoregulation or ITS, across the whole assemblage and between the two families tested individually (Hesperiidae and Nymphalidae) (Table S9).

When data were restricted to the range of air temperatures which occurred in both regions, there was no difference in the use of microclimate selection, postural thermoregulation or ITS between butterflies from neotropical and temperate regions (Results S4; Figure S5 and Table S10). Microclimate selection and ITS did not differ significantly between neotropical and temperate Nymphalidae, but temperate Nymphalidae used postural thermoregulation more than neotropical Nymphalidae (Results S4; Figure S5 and Table S10). There were insufficient data to undertake this analysis for the other families.

4 | DISCUSSION

We found that at the assemblage level, neotropical butterflies are better at buffering their body temperature against changes in air temperature than temperate butterflies. At the family level the same trend was found in Nymphalidae and in the comparison of the three neotropical Riodinidae species to the single European species, but there was no difference for Lycaenidae, Pieridae or Hesperiidae. In the dataset restricted to the range of air temperatures overlapping in both regions, temperate Hesperiidae and Lycaenidae showed better buffering ability than their neotropical counterparts. When controlling for morphological characteristics, there was no difference in buffering ability between the two regions, but larger species and those with higher wing aspect ratios (longer, shallower wings) were better at buffering their body temperature against changes in air temperature than smaller species and those with lower wing aspect ratios (shorter, deeper wings). There were no differences in the use of microclimate selection for thermoregulation between butterflies from neotropical

FIGURE 3 Microclimate selection (the difference between microclimate temperature and air temperature, (a) postural thermoregulation (the difference between body temperature and microclimate temperature, (b) and index of thermal specialisation (postural thermoregulation—microclimate selection, (c) of temperate (blue) and neotropical (pink) butterflies. Significant differences between neotropical and temperate butterflies are marked with an asterisk.



and temperate regions at the assemblage or family level. At the assemblage level, postural thermoregulation was used more to control temperature by temperate than neotropical species, and this was also true for Nymphalidae, but not for Hesperidae, at the family level. Overall, the index of thermal specialisation was greater in temperate butterflies than in neotropical butterflies, but at the family level this was only the case for Nymphalidae and not Hesperidae.

4.1 | Thermal buffering ability

Across the whole assemblage, neotropical butterflies had a better buffering ability than temperate butterflies, with neotropical species having relatively cooler body temperatures at higher air temperatures than temperate species. At the family level, only Nymphalidae and Riodinidae showed a difference in buffering ability between neotropical and temperate regions, with neotropical Nymphalidae and Riodinidae species being better at buffering compared to their temperate counterparts. However, as there was only one temperate and three neotropical Riodinidae species in our dataset, it is not possible to make general assumptions about the thermoregulatory capacities of butterflies from this family, and our finding likely reflects the poor buffering ability of *Hamearis lucina*, the only European species. As half of the species sampled in both temperate and neotropical regions were from the Nymphalidae family (47 of 91 species) it

is likely that this family was driving the trend observed at the assemblage level. Notably, this result was the same when data were restricted to the range of air temperatures which occurred in both regions, indicating that this is a pattern which is independent of the specific range of temperatures butterflies experienced during this study. However, in the restricted dataset, temperate Hesperidae and Lycaenidae species showed better buffering ability than their neotropical counterparts, suggesting that they were better able to cope with changes in air temperature. This result was likely driven by temperate butterflies warming up more at a given air temperature, in an effort to increase activity in the cooler, temperate environment.

The buffering ability response from the dataset including the full range of air temperatures was driven by neotropical species cooling down more than temperate species at high air temperatures. Indeed, at the species level, some neotropical species maintained their body temperature at temperatures lower than air temperature, when temperatures were high (e.g. *Heliconius hecale melicerta*). There is likely to be strong selection among neotropical species to avoid high temperatures, as tropical species live closer to their thermal safety margin (Deutsch et al., 2008; Sunday et al., 2012, 2014, 2019). The better buffering ability of neotropical butterflies may be further explained by differences in habitat type between the neotropical and temperate regions. There was more tree cover and shade in the neotropical sites compared to the temperate sites, and hence a greater availability of cool microclimates. At high air temperatures,

neotropical butterflies may use these marginally cooler microclimates to lower their body temperature, avoiding their thermal limit and the associated decrease in survival and fitness.

It is important to note that our analysis only included data for 54 of 601 neotropical species found in the region (Basset et al., 2015), and 40 of 496 temperate species (Wiemers et al., 2018). This means our results may not be representative of the entire tropical and temperate butterfly assemblages found in the wider regions. However, the neotropical species we sampled are not endemic to Panama and are found elsewhere in the neotropics, making our tropical data likely to be a reliable subsample of the wider neotropical butterfly community. It is also possible that the species we surveyed have a higher than average thermoregulatory ability for their assemblage, as butterflies which thermoregulate efficiently are able to be more active, hence are more likely to be seen and sampled. Nonetheless, we sampled a large pool of species at random, which is likely to reduce the chance of this bias affecting results, and any bias for abundant or conspicuous species is likely to be similar in both regions. Finally, the differences in buffering ability we recorded between regions could be the result of evolutionary history and genetic drift, rather than selection pressures, as neotropical and temperate butterflies diverged a long time ago. However, by undertaking family-level analysis we partly control for phylogeny-driven differences in buffering ability. Additionally, thermoregulation is a highly important factor in butterfly ecology, so traits related to temperature control are likely to be under strong selection pressure.

4.2 | The importance of morphological traits

Alongside the better buffering ability of neotropical species at the assemblage level, we found that neotropical butterflies are generally larger and darker and have a higher wing aspect ratio (longer, shallower wings) than temperate butterflies. Larger species and those with higher wing aspect ratios had a better buffering ability than smaller species and those with lower wing aspect ratios (shorter, deeper wings). This reflects similar results elsewhere. For example, large temperate butterflies buffer air temperature changes better than small butterflies (Bladon et al., 2020), because their larger wing surface area allows them to better regulate their temperature by basking (Kingsolver, 1988; Shanks et al., 2015; Wasserthal, 1975; Xing et al., 2016; Zeuss et al., 2014). Additionally, large butterflies may be more mobile and able to search out cooler microclimates, allowing them to buffer against high air temperatures (Chazot et al., 2016). High wing aspect ratio is associated with greater mobility and flying speed (Hassall, 2015). As far as we are aware it is unknown how wing aspect ratio impacts thermoregulatory ability, however, greater mobility may enable neotropical butterflies to search out favourable microclimates more effectively. Therefore, the better buffering ability of neotropical butterflies compared to temperate butterflies in our dataset could be due to either the larger wing length or higher wing aspect ratio of the neotropical species. However, when these effects were controlled for, the difference in buffering ability between regions did not persist.

4.3 | Microclimate selection, postural thermoregulation and index of thermal specialisation

We found that there was no difference in the use of thermoregulation via microclimate selection between neotropical and temperate species, but temperate species used postural thermoregulation to raise their body temperature more than neotropical species. Neotropical species generally maintained their body temperature closer to ambient air temperature than temperate species. Using postural thermoregulation to heat up is likely to be an adaptation to cooler climates. Butterflies require heat to become active, and lose heat as they fly (Advani et al., 2019). When there is a large difference between air temperature and optimal body temperature, as in temperate systems, heat loss during flight is greater, and so temperate species may be under stronger selection than neotropical species to use postural thermoregulation to heat up, enabling them to become active and increase flight duration in cooler air temperatures. Indeed, temperate butterflies from higher latitudes take-off at higher body temperatures than butterflies from lower latitudes (Advani et al., 2019). For temperate species in our study, undertaking postural thermoregulation to gain more heat before take-off might compensate for the additional heat lost during flight at lower air temperatures, which could increase activity and survival. This result could also be due to other morphological differences between neotropical and temperate butterflies, such as colour, with lighter species, which were found more commonly in the temperate than neotropical study sites, being able to undertake postural thermoregulation better than darker species. Alternatively, it may be a consequence of the different habitat types that were sampled between neotropical and temperate regions. In the temperate region more open habitats were sampled, which would have provided greater opportunities for basking and could explain why temperate butterflies undertook postural thermoregulation more than neotropical butterflies.

We found that neotropical species were more thermally specialist, relying relatively more on the selection of available microclimates than postural thermoregulation, when compared to temperate species. However, this result is driven by temperate species undertaking postural thermoregulation to increase their body temperature. Neotropical species maintained their body temperature closer to ambient temperature than temperate species did, especially at higher air temperatures, presumably as a mechanism to avoid overheating. This could make them vulnerable to extreme heatwave events, as, if air temperature increases, their ability to avoid temperatures outside their thermal safety margin might become limited.

At the family level, we found the same result in Nymphalidae, the most abundant family in our dataset, but not Hesperidae, so Nymphalidae were likely driving the assemblage-level trend. Nymphalidae species are often larger than those in other butterfly families, and it is possible that their wing size enables them to undertake postural thermoregulation more effectively, as larger wings can better control temperature through basking than smaller wings.

4.4 | Implications

Climate change, through increasing temperatures, is expected to negatively affect ectotherms, decreasing their performance and, in extreme cases, resulting in extinction (Diamond & Yilmaz, 2018; Johansson et al., 2020; Mi et al., 2022). By buffering their body temperature against changes in air temperature using a combination of microclimate selection and postural thermoregulation, butterflies might be able to mitigate some of these negative effects. We found that neotropical butterfly species, which may be living closer to their thermal limit than temperate species, buffer their body temperature against changes in air temperature better than temperate species, primarily by cooling down more at higher air temperatures (Deutsch et al., 2008; Sunday et al., 2012, 2014, 2019). In the restricted dataset, temperate Hesperidae and Lycaenidae were better at buffering their body temperature against changes in air temperature than their neotropical counterparts, but this was driven by their ability to warm up more at a given air temperature. Similarly, temperate butterflies, and in particular Nymphalidae, were better at using behaviour for thermoregulation than neotropical butterflies, but this is likely to be an adaptation for warming up in cool temperate climates, and may not indicate a good ability to cool down under rising temperatures. Therefore, both neotropical and temperate butterfly species show signs of vulnerability to rising temperatures, with no overriding ecological difference in sensitivity to climate change between bioclimatic regions. However, the degree to which physiological acclimation or genetic adaptation could offset the negative effects of increasing temperatures on these butterflies remains unclear (Diamond & Yilmaz, 2018; Seebacher et al., 2015; Sgrò et al., 2016; Sheldon & Tewksbury, 2014).

Our study has focused solely on the adult stage, but thermal sensitivity and hence vulnerability to changing temperatures varies markedly between life stages (Kingsolver et al., 2011). Indeed, life stage is the most important factor for predicting tolerance to changes in temperature, and mortality mostly occurs before the adult stage (Bowler & Terblanche, 2008). Eggs are thought to be the most vulnerable life stage, compared to larvae, pupae and adults, as a consequence of their physiology and their immobility, which means they cannot locate more favourable microclimates (Kingsolver et al., 2011). Larvae are also likely to be sensitive to increasing temperatures, as although mobile, their dispersal ability is limited (Kingsolver et al., 2011). Additionally, most Lepidoptera spend more time in the larval than the adult stage, meaning temperature changes are more likely to impact this life stage. Therefore, future studies should investigate the buffering ability and thermoregulatory mechanisms of all butterfly life stages.

Although microclimates may offer thermal refugia for adult Lepidoptera, the extent to which they will benefit less mobile life stages with regards to temperature control and coping with temperature extremes remains unclear (Montejo-Kovacevich et al., 2020). Habitat degradation and land-use change has been shown to decrease the availability of cooler microclimates and hence the

temperature buffering potential of a habitat (Jucker et al., 2020). As conversion of land for agricultural use continues to accelerate and global temperatures rise, the protection of pristine habitats for their temperature buffering potential may become increasingly important.

5 | CONCLUSION

To our knowledge, this is the first study comparing the thermal buffering ability of ectotherms between tropical and temperate systems. By using standardised methods to measure buffering ability and mechanisms, we provide a template for making cross-latitudinal comparisons within taxa, increasing understanding of how species across latitudes cope with changing temperatures. We stress the need for further cross-latitudinal studies comparing the thermal capabilities of tropical and temperate species. Despite over half of the world's insect species living in tropical systems, most research focuses on the thermal capabilities of temperate ectotherms (García-Robledo et al., 2020; Stork, 2018). Future studies should focus on tropical ectotherms and expand knowledge of how these species regulate temperature and their potential vulnerabilities to climate change. We also highlight the importance of considering species' morphological traits, and use of microclimate selection and postural thermoregulation when predicting the vulnerability of tropical and temperate species to future climate change. Future studies should also investigate the role of habitat structure in the buffering ability of butterflies, for example determining how favourable microclimates may act as refuges from extreme temperatures for tropical and temperate species, paving the way to informing management options that could protect species from warming. Most importantly, our results show that individual species have their own individual thermoregulatory capabilities. We stress the need for caution when making generalised assumptions of species' vulnerability to climate change based solely on the thermal environment they inhabit.

AUTHOR CONTRIBUTIONS

The study was designed by Andrew J. Bladon, Edgar C. Turner, Benita C. Laird-Hopkins and Esme Ashe-Jepson. Data were collected by Benita C. Laird-Hopkins, Esme Ashe-Jepson, Irena Kleckova, Andrew J. Bladon, Gwen E. Hitchcock, Josh Hellon, Ana Cecilia Zamora, Amanda F. Savage, Alex McFarlane, Stephany Arizala Cobo, Daniel Linke, Inga Freiberga and Lucy Eberhardt. Benita C. Laird-Hopkins conducted the analyses and wrote the manuscript. All authors contributed substantially to revisions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Zenodo at <http://doi.org/10.5281/zenodo.7900256>.

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SUPPORTING INFORMATION

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Appendix B

Curriculum Vitae

Benita C. Laird-Hopkins

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Education

Ph.D Entomology. **University of South Bohemia/Czech Academy of Sciences** 2019-present

- Supervisor: Dr. Katerina Sam
- Butterfly thermoregulation across habitats and climates.
- Smithsonian Tropical Research Institute (STRI) Visiting Predoctoral Fellow

M.Sc Ecology. **Lancaster University** 2015-16

- Supervisor: Dr. Emma Sayer; Co-supervisor: Dr. Laetitia Brechet
- Tree functional diversity affects litter decomposition and arthropod community composition in a tropical forest.
- Independently designed and set up the experiment in Panama.
- Analysed soil and arthropod samples; Experienced in statistical analysis and writing manuscripts.

B.Sc (Hons) Biology. **The University of Bristol**. First class 2011-14

Professional Appointments

Teaching Assistant. **McGill University and STRI**, Panama 04-05.2022

- Assisted in teaching and organising a four-week field course for ecology and sociology university students.

Lead instructor. **Wildlands Studies**, Alaska, USA 04-09.2019

- Taught a six-week ecology field course to university students.
- Responsible for developing the syllabus, designing the itinerary, organising logistics, managing the budget and teaching the course.

Ranger. **Department of Conservation**, New Zealand 2018-19

- Participated in bird surveys and undertook stoat and possum trapping.
- Disseminated information on conservation issues and biodiversity through presentations, interpretive walks and workshops to members of the public.

Research Associate/Project Manager. **Iowa State University**, Guam 2017-18

- Supervisor: Dr. Haldre Rogers, Iowa State University. In collaboration with Dr. Yves Basset, STRI
- Worked as part of the Ecology of Bird Loss research group.
- Responsibilities included training and supervising field assistants; planning and coordinating field work across islands; organising all logistical tasks.
- Designed and set up a project comparing inter-island insect seed predator networks and investigated the effect of bird loss on the insect seed predator community.
- Part of an international latitudinal comparison project of seed predator communities and a major contributor to the entomology collection of Guam and Saipan.

Project Manager. **STRI**, Panama 2016-17

- Supervisors: Prof. Vojtech Novotny, Czech Academy of Sciences; Dr. Yves Basset, STRI
- Inter-institutional collaboration between the Czech Academy of Sciences and the STRI that used quantitative surveys of plant-herbivore-parasitoid food webs to understand why so many species can coexist in tropical forests versus temperate forests.
- Duties included coordinating the Panamanian section of a €3m international research project; managing a multi-cultural team of 15 researchers from ten different countries; sampling trees for miners, gallers and caterpillars using a canopy crane and by climbing trees; morphotyping and rearing miner, galler and caterpillar samples; advanced entomological ID skills; responsible for financial and administrative tasks.

- Research Assistant. **STRI, Thailand** 2014
- Worked on the CTFS Arthropod Initiative with the aim to monitor arthropod diversity.
 - Set up and managed an experiment looking at fruit and seed density.
 - Advanced plant and arthropod identification skills; prepared samples for DNA analysis and export.
- Volunteer. **Bristol Natural History Consortium, UK** 2014
- Monitored plants, lichen, arthropods and fish in terrestrial and aquatic habitats.
 - Communicated science to the public and taught young people biological surveying techniques.
- Research Administrator. **University of the West of England, UK** 2010-14
- Administrative support for the Keeping Children Safe at Home research team, a National Health Service funded research project investigating how to increase child safety in the home.
- Researcher/Backcountry trail builder. **National Park Service, Alaska, USA** 2013-14
- Surveyed plant and pollinators; Organised and undertook backcountry patrols.
 - Built trails; experienced in using and maintaining power and hand tools and driving ATV's off road.
 - Managed a remote backcountry cabin, provided visitors with information and disseminated bear safety training.
- Field and Lab Assistant. Community Ecology Lab/The Ant Lab. **University of Bristol, UK** 2012-13
- Conducted pollinator surveys and prepared insect samples for identification.
 - Independently designed and undertook experiments investigating decision making between ants involved in tandem runs; solely responsible for the project.

Awards

- Student presentation awards, second place, University of South Bohemia, Jan 2021, Jan 2022 and Feb 2023
- Student poster prize, highly commended, Festival of Ecology, British Ecological Society, Dec 2020
- Travel award, University of South Bohemia, Jan 2020
- Short-Term fellowship, Smithsonian Tropical Research Institute, April 2020, \$4000

Publications

- **Laird-Hopkins, B. C., et al.,** (in review). Regional differences in thermoregulation of European butterflies is driven by temperature, precipitation and geographic region. *Biological Communications*
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- **Laird-Hopkins, B. C., et al.,** (2023). Thermoregulatory ability and mechanism do not differ consistently between neotropical and temperate butterflies. *Global Change Biology*, 29, 4180-4192
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Posters and Presentations

- **Laird-Hopkins, B. C., et al., (2023).** Thermoregulation in butterflies across habitat types, bioclimatic regions and latitudes. The University of Sheffield. April. [Presentation](#)
- **Laird-Hopkins, B. C., et al., (2023).** Thermoregulatory ability and mechanism do not differ consistently between tropical and temperate butterflies. University of South Bohemia. Feb. Czech Republic. [Presentation](#)
- **Laird-Hopkins, B. C., et al., (2022).** Tropical butterflies may be more sensitive to climate change than temperate butterflies. *Festival of Ecology, British Ecological Society*. Dec. [Poster](#)
- **Laird-Hopkins, B. C., et al., (2021).** The thermoregulatory abilities of *Arashnia levana*. University of South Bohemia. Feb. Czech Republic. [Presentation](#)
- **Laird-Hopkins, B. C., et al., (2020).** Long-term population trends of adult Lepidoptera in a tropical forest: Barro Colorado Island, Panama. *Festival of Ecology, British Ecological Society*. Dec. Virtual. [Poster and presentation](#)
- **Laird-Hopkins, B. C., et al., (2020).** In a changing climate: the thermal and desiccation tolerance of tropical butterfly communities. *Czech Academy of Sciences*. June. Czech Republic. [Presentation](#)
- **Laird-Hopkins, B. C., et al., (2019).** In a changing climate: the thermal and desiccation tolerance of tropical butterfly communities. *Smithsonian Tropical Research Institute*. November. Panama. [Presentation](#)
- **Laird-Hopkins, B. C., et al., (2017).** The effect of bird pollinator-loss on the reproductive success of plant species. *Marianna Island Terrestrial Conference*. Nov. Guam. [Poster](#)
- **Laird-Hopkins, B. C., (2017).** Ecological determinants of tropical-temperate trends in insect diversity. *Princeton University tropical field course*. Feb. Panama. [Presentation](#)
- **Laird-Hopkins, B. C. and Beckett, S., (2016).** Biodiversity in Tropical Forests. *CEASPA*. Sep. Panama. [Presentation](#)
- **Laird-Hopkins, B. C., et al., (2015).** Tree functional diversity affects litter decomposition and arthropod community composition in a tropical forest. *Soil Ecology Annual Meeting*. Dec. Lancaster, UK. [Presentation](#)
- **Laird-Hopkins, B. C., et al., (2015).** Linking soil respiration to decomposition of litter functional groups in a tropical forest. *British Ecology Society Plants, Soils, Ecosystems and the Climate Change Ecology*. Nov. London, UK. [Poster](#)
- **Laird-Hopkins, B. C., et al., (2015).** Linking soil respiration to decomposition of litter functional groups in a tropical forest. *British Ecology Society Annual Meeting*. Dec. Edinburgh, Scotland. [Poster](#)

Skills

- Languages: English, Spanish, German (A2)
- Statistics: ANOVA, GLM, LME, phylogenetic analysis, data visualisation
- Software: R statistical software, QGIS, Adobe Photoshop, Office suite
- Field skills: bird, plant and arthropod identification, bird netting and health checks, telemetry
- First aid: Pre-Hospital Emergency Care (2019)
- Other: driving licence, boat driving license, rock and tree climber, PADI Advanced Open Water