Exploring phenotypic and transcriptomic responses of *Heliconius* butterflies to temperature across altitudes and between species

Tien T. T. Nguyen

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Declaration

The work presented in this thesis was performed at the University of Sheffield between October 2019 and December 2023 under the supervision of Dr. Nicola Nadeau. Unless stated otherwise, the work is that of the author. No part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution.

Tien T. T. Nguyen

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Abstract

In the face of climate change, understanding temperature response and adaptation remains a continuously relevant subject in eco-evolutionary biology, especially in insects. The tropics, which hold the wealth of the world's natural biodiversity, are thought to be the most vulnerable to rising and unstable temperatures, with species currently living close to their upper thermal limits. Altitudinal clines provide natural temperature gradients, excellent for studying how temperature influences intraspecific variation and the underlying mechanisms governing thermal tolerance and response. *Heliconius* butterflies are neotropical and can be found along the altitudinal slope of the Andes, and here I present them with great potential as 'neotropical models'. Having been extensively studied for their colour patterning and mimicry, they boast numerous genomic resources, yet their thermal adaptations have been relatively understudied. In this thesis, (1) I use *Heliconius sara*, a heat hardy species reared in extreme but field-relevant temperatures to provide foundational insights into *Heliconius* thermal response across three life stages, larvae, pupae, and adults in gene expression as well as their development and thermal tolerance. 2) I then use altitudinal populations of mimetic species *Heliconius erato* and *Heliconius melpomene* reared in controlled environments to disentangle local adaptation and phenotypic plasticity in traits associated with altitude and thermal adaptation. 3) Finally, to discern the interplay between local adaptation and plasticity in gene expression responses to thermal stress. I utilized *Heliconius erato* samples from high and low altitudes, reared in their reciprocal temperature environments. By combining rearing experiments with transcriptomics, I aimed to effectively bridge the gap between phenotype and genotype, offering a comprehensive understanding of how species adapt

and respond to temperature. This holistic approach also provides valuable insights into how species respond to future climate change scenarios.

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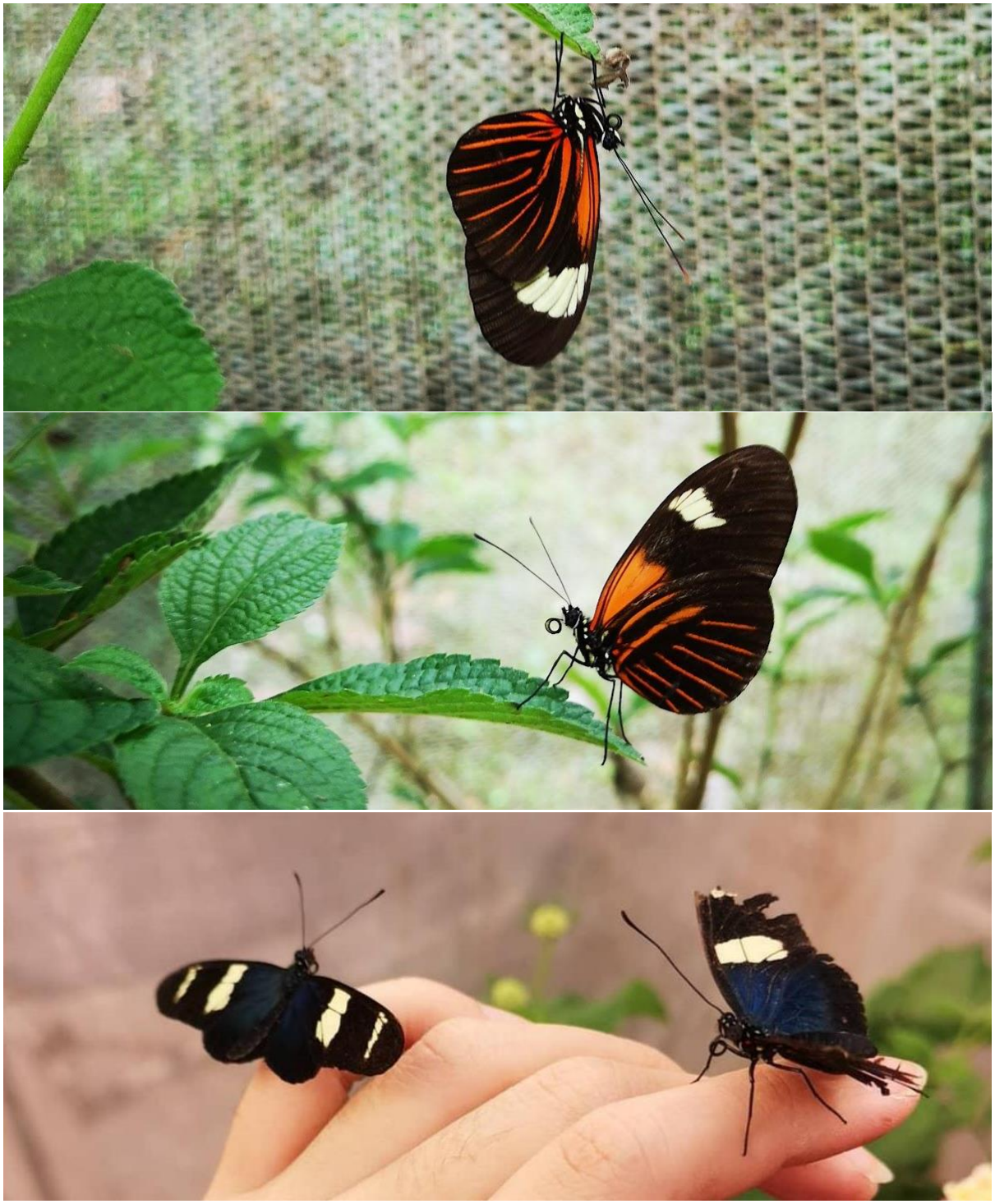
Table of Contents



A wild and captive study across the globe. Top, *Heliconius erato*, middle, *Heliconius melpomene* wild caught specimens taken at Universidad Regional Amazónica Ikiam, Tena, Ecuador. Bottom, *Heliconius sara* captive stock butterflies, taken at the University of Sheffield, UK.

Chapter 1

General Introduction

Temperature is a critical ecological factor that drives evolution (Foster and Rahmstorf, 2011). It can shape the life history of organisms by impacting behaviour, physiology, and geographical distribution. Thus, adaptation to temperature heterogeneity is paramount for survival (Buckley and Kingsolver, 2021). The advent of climate change is anticipated to bring not only elevated average temperatures, but also heightened temperature variability and an increased frequency of extreme events (Radchuk *et al.*, 2019). Therefore, understanding how species respond, and the underlying mechanisms of their thermal adaptations will continue to be increasingly relevant, especially in the context of insects (Harvey *et al.*, 2023). This is because insects play a pivotal role in maintaining ecosystem stability due to their low trophic level (Gross *et al.*, 2005; Van Der Putten, Macel and Visser, 2010). Here we focus on *Lepidoptera*, the second-largest insect order. These insects hold substantial socio-economic importance, encompassing a myriad of captivating species that carry significance, in culture, and food security in relation to ecosystem services but also as potential pests (Goldstein, 2017; Scudder, 2017; Deutsch *et al.*, 2018; Basset and Lamarre, 2019).

The majority of insect temperature research has centred around *Drosophila* (Kellermann and van Heerwaarden, 2019) or focused on temperate species (Hodkinson, 2005), with few exceptions such as Afrotropical butterfly *Bicyclus anynana* (Fischer *et al.*, 2010). Yet, it is thought that tropical species (Deutsch *et al.*, 2008; Bonebrake and Deutsch, 2012; Chowdhury, 2023) are the most vulnerable to climate change, due to the lack of seasonality in the tropics, resulting in a narrow range for temperature selection (Janzen,

1967; Bonebrake and Deutsch, 2012). Thus, even though the rate of rising temperatures is thought to be lesser in the tropics, the magnitude of its effect will be much higher in comparison to temperate counterparts. Furthermore, it is also thought that tropical species are already currently living close to their upper thermal limits (Deutsch *et al.*, 2008; Bonebrake and Deutsch, 2012).

Temperature adaptation in the tropics

To maintain homeostasis and effectively thermoregulate, Lepidopterans employs a range of adaptive strategies (Mutamiswa *et al.*, 2023). These encompass behavioural, physiological, and morphological adaptations, often operating in combination (González-Tokman *et al.*, 2020).

Behavioural mechanisms are probably the first to be utilised in thermal response and adaptation. They include basking behaviour, microhabitat selection, modifications of activity cycle and even migration (Masters, Malcolm and Brower, 1988; Shreeve, 1990; Bladon *et al.*, 2020). Tropical butterfly species have been shown to increase basking behaviour at cooler temperatures (Wenda *et al.*, 2021), as well as making use of microhabitats to maintain optimal body temperatures (Montejo-Kovacevich *et al.*, 2020; Wenda *et al.*, 2021). It is thought that in the lowland tropics, species greatly rely upon behavioural mechanisms to prevent overheating (Sunday *et al.*, 2014). Therefore, the presence of diverse microclimates is thought to be particularly beneficial in tropical forests, allowing for buffering against future climate change scenarios (Storlie *et al.*, 2014). However, rising temperatures have been shown to cause range shifts in the tropics (Molina-Martínez *et al.*, 2016), and eventual limitations associated with suitable habitat

availability may prove to be detrimental to species without other mechanisms of thermal adaptation (Berg *et al.*, 2010).

The significance of body size in thermal adaptation has been a subject of frequent consideration. It has often been observed that as temperatures rise, body size tends to decrease, a phenomenon often explained by two associated rules: Bergmann's rule (1848) and the temperature-size-rule (Atkinson, 1994). While Bergmann's rule (1848) has been used to describe an adaptive association between temperature and body size, the temperature-size-rule describes reaction norms relating temperature to body size observed in laboratory experiments, i.e a plastic response (Atkinson, 1994). Bergmann's rule (1848) suggests an adaptive advantage involved with thermal regulation and tolerance, with the idea of larger bodies allowing an individual to better buffer against the cold environment, while smaller individuals in warmer environments would have more control over the heat exchange with the external environment, thus prevent overheating. However, this rule was originally used to describe such phenomena in endotherms interspecifically (Blackburn, Gaston and Loder, 1999) (but was later adapted intraspecifically also (James, 1970; Blackburn, Gaston and Loder, 1999)), while insects have shown conflicting results, with some following the rule (Horne, Hirst and Atkinson, 2018; Svensson, Gómez-Llano and Waller, 2023) and others following the reverse trend (Mousseau, 1997). This discrepancy has brought into question whether Bergman's rule (1848) offers an adaptive advantage in insects (Partridge and Coyne, 1997; Shelomi, 2012). For example, in the tropical butterfly *Hypolimnas bolina*, it was found that smaller males had an advantage in warmer environments by utilising a greater number of basking postures to control heat radiation and absorption (Kemp and Krockenberger, 2004) therefore, would follow the rule. Whereas in *B. anyana* larger body sizes (with respect to

reducing surface area to volume ratio) increased their heat tolerance (Klockmann, Günter and Fischer, 2017) thus showing adverse trends in the adaptive advantage of body size in thermoregulation and tolerance. This also suggests that the patterns of body size in ectotherms may depend on the type of temperature selection pressure, with both duration and magnitude. Furthermore, a meta-analysis across ectothermic species by Peralta-Maraver and Rezende (2020) found that ectothermic heat tolerance could be predicted with body size, where smaller animals tended to be able to maintain higher body temperatures than larger ones during short periods. However, smaller animals could not maintain higher body temperatures over long periods, as their endurance declines more rapidly with time, in comparison to larger individuals.

The temperature-size-rule (Atkinson, 1994) in laboratory experiments has been observed across species, where higher rearing temperatures reduce the time allowed to develop, therefore leading to smaller body sizes. This plasticity is thought to be a trade-off with growth rate, and development time (Kingsolver and Huey, 2008), where larger body sizes have often been correlated with a high competitive ability as well as high fecundity. Furthermore, life history models typically assume that fitness increases continuously with adult size (Fischer *et al.*, 2003; Berger, Walters and Gotthard, 2008). Whereas shorter development time supposedly reduces vulnerabilities in juveniles and allows for faster dispersal therefore, improving competitive ability for resources and mates (Rolff, Johnston and Reynolds, 2019). However, the temperature-size-rule is not universal (Angilletta and Dunham, 2003) including in *Lepidoptera* (Steigenga and Fischer, 2009) and could instead be associated with optimal developmental growth temperatures where smaller bodies may suggest a degree of stress in particular developmental temperatures (Forster and Hirst, 2012).

A common morphological phenomenon associated with thermal adaptation observed across the globe including the tropics, is that of colouration with regard to the thermal melanism hypothesis, which suggests that colouration is associated with environmental temperature in aid of thermoregulation (Clusella Trullas, van Wyk and Spotila, 2007). This has been observed in Australasian butterflies where darker species were found in cooler environments (Xing *et al.*, 2016) and in neotropical *Catastica* butterflies, which were darker with increased altitudes (where it is colder) (Dufour *et al.*, 2018). The increased darkening was linked to their thermal regulation as the darker colouration allowed greater heat and radiation absorption. Monarch butterflies have been shown to have induced thermal melanism associated with thermoregulation, showing that thermal melanism can have a degree of adaptive plasticity (Davis, Farrey and Altizer, 2005).

Acclimation is a common physiological response in thermal adaptation, where according to the beneficial acclimation hypothesis (Leroi, Bennett and Lenski, 1994; Wilson and Franklin, 2002), provides an organism with advantages where mild exposure to temperature stress can aid in survival when exposed to extremes later on. This has been observed across both temperate (Karl *et al.*, 2008) and tropical species (Fischer *et al.*, 2010). Furthermore, proteomic studies in *Drosophila* suggested through links with chromatin modifications and epigenetics, that acclimation (Colinet *et al.*, 2013) may have a degree of inherited plasticity, where thermal experiences from the mother may prime offspring to be resilient to the thermal stress experienced by their mothers (Fox and Mousseau, 1998; Zhou *et al.*, 2013; Gu *et al.*, 2019).

However, it has been reported that tropical species possibly have lower acclimation rates in comparison to temperate species (Bonebrake and Deutsch, 2012) again emphasizing the concerning vulnerability of tropical species to changing environments. Typically, cold

acclimation is associated with antifreeze proteins and or cryoprotectants (Duman and Newton, 2020). However, these are not likely to be relevant in tropical systems as freezing is not necessarily a concern in tropical systems, unless they occupy both temperate and tropical spaces, and or are upper montane species. Therefore, the physiological and molecular mechanisms underpinning cold tolerance have not been extensively studied in tropical models and have even been suggested to be of lesser importance (Sørensen *et al.*, 2005). However, it should be considered that thermal adaptations can overlap, particularly with acclimation, where cold acclimation has been shown to also increase heat tolerances (Hemmati *et al.*, no date; Sejerkilde, Sørensen and Loeschcke, 2003; Colinet *et al.*, 2013). This is thought to be because of the overlap in the consequences of thermal stresses, such as desiccation (Rajamohan and Sinclair, 2008; Sinclair *et al.*, 2013). In heat acclimation, heat shock proteins (hsps) are often centred where they are seen to up-regulate at faster speeds and higher concentrations (Sejerkilde, Sørensen and Loeschcke, 2003). Heat shock proteins are stress proteins that are not necessarily specific to temperature but have been extensively studied in regard to heat adaptations (Zhao and Jones, 2012). They are typically protein chaperones that fold, re-fold or prevent unfolding to minimalize and or reverse the effects of protein denaturing caused by high heat (Zhao and Jones, 2012). Hsp70, in particular, has been linked closely with thermal adaptation, where it is involved in acclimation (including cold tolerance (Sejerkilde, Sørensen and Loeschcke, 2003)) and heat tolerance under extreme temperatures (Krebs and Bettencourt, 1999). However, what hsp70 expression truly indicates in heat tolerance has shown differences between species. For example in neotropical *Drosophila buzzatii*, thermal tolerance was observed to decrease with increasing altitudes, which was expected due to the colder environment, while their hsp70 levels increased (Sørensen *et al.*, 2005) supporting Sørensen, Dahlgaard and

Loeschcke's (2001) previous claim that increased hsp70 levels indicate increased stressful conditions as hsp70 had also been reduced with pre-acclimation. Yet the temperate butterfly, *Lycaena tityrus*, showed similar patterns in heat tolerance with altitude, but hsp70 expression was greater in lowland populations than in highlands (Karl *et al.*, 2009). This difference may be attributed to the cost and benefit of maintaining Hsp expression, given their energetic demands (Feder *et al.*, 1992; Krebs and Loeschcke, 1994). It may indicate differences in priority, evolutionary history (temperate vs tropical), or possible additional mechanisms allowing greater expression in *L. tityrus* (Karl *et al.*, 2009) and or reduced expression in *D. buzzatii* (Sørensen, Dahlgaard and Loeschcke, 2001; Sørensen *et al.*, 2005). Hence, this emphasises the importance of finely-tuned research into thermal adaptation. Furthermore, it has also been suggested that aside from hsps, we know little about the genetic basis of heat adaptations (González-Tokman *et al.*, 2020).

Temperature adaptation across environmental gradients

Environmental gradients provide valuable opportunities for investigating species' evolution and adaptation to changing environments, as variation along these environmental clines in traits related to fitness suggests directional selection (Doebeli and Dieckmann, 2003; Tylianakis and Morris, 2017; Riesch, Plath and Bierbach, 2018). Both latitude and altitude offer temperature gradients that enable us to study species' responses to temperature variation (Körner, 2007; De Frenne *et al.*, 2013; Riesch, Plath and Bierbach, 2018).

Latitudinal studies capture broad climatic trends around the globe, allowing for comparative studies across a wide range of ecosystems, and providing information on how different species and communities respond to temperature and stress resilience (De

Frenne *et al.*, 2013). With regard to thermal tolerance, it has been generally observed that thermal limits are wider with increasing latitudes, particularly in the Northern Hemisphere, which is thought to be linked to the greater seasonality (Janzen, 1967; Sunday, Bates and Dulvy, 2011). However, this is specifically with lower limits which decline dramatically with increasing latitudes, while upper limits have been found to be relatively similar (Addo-Bediako, Chown and Gaston, 2000; Sunday *et al.*, 2019). This suggests vulnerabilities to future climate change due to constrained upper limits. However, it is important to consider not only phenotypic variation but also genomic, which allows deeper insight into evolutionary capacity in developing thermal tolerances. For example, Australian populations of *D. melanogaster* have shown DNA sequence variations in hsp26 in association with latitude and stress response (Frydenberg, Hoffmann and Loeschcke, 2003).

Although latitudinal studies enable potential assessments of global trends, there is strong caution advised against assuming that macroecological patterns can be universally applied across various systems (Paine, 2010). This is because temperature patterns at particular latitudes can be influenced by various factors, including local geography and altitude (De Frenne *et al.*, 2013). Therefore, this complexity can make it challenging to disentangle the true effects of temperature. For instance, the presence of diverse microclimates, particularly in tropical forests, can introduce buffering effects (Storlie *et al.*, 2014), which was emphasised in Australian alpine butterflies in genus *Erebia*, which utilize microhabitats in their thermoregulatory strategies (Kleckova, Konvicka and Klecka, 2014).

In contrast, altitudinal studies cover relatively short distances with well-documented geography, providing a more focused insight into temperature effects (Körner, 2007).

This detail is crucial in understanding local adaptations across populations, particularly through common garden and reciprocal transplant rearing experiments which help determine whether observed adaptations result from genetic adaptation, plasticity, or a combination of both with a genotype-by-environment interaction (GXE) (Fox *et al.*, 2019). For example, *L. tityrus* showed evidence of altitudinally linked thermal adaptation, as when altitudinal populations were reared at the same rearing temperature, highland compared to lowland populations were shown to have longer development time, increased cold, but decreased heat-stress resistance, and increased flight duration (Karl *et al.*, 2008). This increased heat-stress resistance in lowland populations was likely attributed to the increased hsp70 expression in lowland populations (Karl *et al.*, 2009). Whereas, the wing solar absorptivity of *Colias eriphyle* which expectedly increased with altitude, where there is more solar radiation and colder atmospheric temperatures, was due to plasticity, dependent upon developmental temperature (Kingsolver and Buckley, 2017). A GXE interaction has been observed in *Melitaea cinxia* with regard to metabolic rates (Niitepõld, 2010) and in *B. anynana* with developmental growth rates (Brakefield and Kesbeke, 1997) where in both species, the trait present is dependent upon the population in interaction with rearing temperature.

Neotropical model *Heliconius*

Here, I propose *Heliconius* as a neotropical model for understanding thermal adaptation and response. *Heliconius* butterflies have been extensively studied for their myriad of colour patterning and Müllerian mimicry, for which they are famous, and therefore boast many genomic resources (The *Heliconius* Genome Consortium, 2012). Furthermore, being widespread across the neotropics and notably along the slopes of the Andes (an altitudinal gradient), they serve as a valuable proxy for studying temperature adaptations.

Thermal adaptations in *Heliconius* butterflies have been relatively understudied. Altitudinal differences have been observed in *Heliconius melpomene* and *Heliconius erato*, indicating a degree of natural selection among populations at varying altitudes (Nadeau *et al.*, 2014; Montejo-Kovacevich *et al.*, 2022). Differences in their wing aspect ratio have also been shown to change across altitudes (their wings were rounder with increasing altitudes), which was suggested to be linked to flight performance at higher altitudes where atmospheric pressure drops (Montejo-Kovacevich *et al.*, 2019; Montejo-Kovacevich *et al.*, 2021). Their heat tolerance appears to be relatively plastic, as the decreased heat tolerance with increased altitude appeared to be lost when offspring from wild-caught mothers from high and low altitudes were reared in semi-outdoor environments in 'common garden', lost this difference (Montejo-Kovacevich *et al.*, 2020). However, Nadeau *et al* (2014) and Montejo-Kovacevich *et al* (2021) showed that there was a degree of natural selection occurring across altitudinal populations of *Heliconius erato* in the locus region encoding for hsp70.

Outline of thesis chapters

While thermal adaptation is thoroughly studied in temperate regions, little is known in tropical species. In this thesis, I aim to explore the mechanisms that underpin *Heliconius* thermal response in their life history and gene expression. I present *Heliconius* as an ideal neotropical model for understanding thermal adaptation through conducting this holistic investigation that bridges the gap between phenotype and genotype (with transcriptomics).

In chapter 2, I describe the effect of extreme but field-relevant rearing temperatures on heat hardy species *Heliconius sara* on their life history and thermal tolerance.

Furthermore, I also describe how extreme rearing temperatures affects their gene expression across three significant life stages: 5th instar larvae, pupae, and adult butterfly, as well as post-exposure to thermal stress in adult butterflies. This non-wild population provides us with a foundation to understanding thermal adaptations in *Heliconius* and the sensitivities of thermal response and acclimation. The large number of differentially expressed genes between the two rearing temperatures, and significantly enriched GO terms associated with temperature response provided us with insight into the molecular underpinnings associated with thermal tolerance in *Heliconius*.

In chapter 3, I discerned altitudinally linked developmental and thermal tolerance traits as well as their heritability from the offspring of *Heliconius erato* and *Heliconius melpomene* mothers caught along an altitudinal gradient that were reared in a 'common garden environment'. I also used populations from high and low altitudes reared in their reciprocal temperature environments to disentangle the nuances of thermal adaptation and phenotypic plasticity.

In chapter 4, through a transcriptomic approach, we unravel the intricate interplay of genes and temperature to uncover the unique molecular strategies that underpin the local adaptation in the *H. erato.* These *H. erato* samples were from high and low altitudes were reared in their reciprocal environments and exposed to extreme thermal stress from chapter 3.

Finally, in chapter 5 I discuss the main findings of this thesis, and suggest future directions for further research building on from this work.

Note on contributions and acknowledgements

Within each chapter, I have made note of contributions and acknowledgements. However, I would also like to acknowledge with full transparency that I have used generative AI (ChatGPT) in aid of correcting R code and shell scripts during analysis. I have also used ChatGPT and Grammarly (plugin) as writing tools to formulate ideas and aid in sentence structuring, as well as to check grammar and punctuation.

Chapter 2

The effect of rearing temperature on development, thermal tolerance and gene expression in the tropical butterfly *Heliconius sara*.

Tien T. T. Nguyen[1], Imogen Elliott[1], and Nicola J. Nadeau[1]*

[1] School of Biosciences, University of Sheffield, Alfred Denny Building, Western bank, Sheffield S10 2TN, UK

*Corresponding author

Email: n.nadeau@sheffield.ac.uk

Author contributions

This chapter was written in a manuscript format for future submission. Myself, Nicola Nadeau and Imogen Elliott designed the research project. Butterfly rearing and thermal tolerance were conducted by myself and Imogen Elliott. I extracted all the RNA for all samples. I wrote the manuscript with feedback from Nicola Nadeau, who also contributed to data interpretation.

Abstract

With the onset of climate change, temperature instability will only continue to worsen, and it is thought that tropical species may be the most vulnerable, yet are relatively understudied. Neotropical *Heliconius* butterflies are well studied for their colour patterning and colouration, yet mechanisms underpinning thermal tolerance and adaptation have been understudied. Here we investigate the effect of two extreme but field-relevant temperatures, on the heat-hardy species *Heliconius sara,* on development and the phenotypic response of adults to extreme cold or heat stress. We also tested the differential gene expression of 5th instar larvae, pupae, and thermally tested adults using RNA sequencing. Some of the genes differentially expressed between rearing temperatures are shared between developmental stages, particularly larvae and adults, but larger numbers are unique to each stage. This suggests that temperature response differs depending on life stages and may denote unique response mechanisms by developmental stage. Adults showed evidence of acclimation in response to thermal stress where colder rearing temperatures led to increased cold tolerance (quicker chill coma recovery times) and warmer rearing temperatures led to increased heat tolerance (longer time to heat comas). Gene set enrichment analysis (GSEA) on up-regulated genes in cooler rearing temperatures suggested that cold acclimation may be associated with cell-wide upstream processes. Rearing temperature did not affect heat tolerance, suggesting that heat acclimation perhaps requires more extreme temperatures or the underlying mechanisms of heat response may have consequences when expressed long term. The GSEA in up-regulated genes found in heat-tolerant adults showed great downstream specificity with enriched GO terms tightly linked to the up-regulation of heat shock proteins, and perhaps suggest heat tolerance is constrained to particular pathways.

However, with no apparent effect of cold acclimation on heat tolerance provides a positive implication for an inevitably changing and more unpredictable climate.

Introduction

Understanding how organisms respond to temperature has always been of great eco-evolutionary interest and with the onset of global climate change, temperature adaptation holds increasing relevance with implications for future scenarios (Hoffmann and Sgró, 2011). Insects in particular are a great system for understanding thermal response due to their particular sensitivities (Robinet and Roques, 2010; Colinet *et al.*, 2015).

Typically, in insects, elevated temperatures within optimal developmental limits often lead to increased developmental rates, which ultimately leads to decreased body sizes and vice versa for lower rearing temperatures. This produces a reaction norm that conforms to the temperature-size-rule (Atkinson, 1994). Whether this plasticity is adaptive has been contested (Partridge and Coyne, 1997; Shelomi, 2012), as developing faster or being larger has associations with advantages and disadvantages. Faster developmental times could allow for faster lifecycle turnover rates, allowing for better competition for resources such as mates and food as adults (Rolff, Johnston and Reynolds, 2019). Whereas, slowed developmental times have been associated with larger and higher quality offspring (Fischer *et al.*, 2003; Fischer, Brakefield and Zwaan, 2003; Berger, Walters and Gotthard, 2008). However, have greater risks associated with predation and environmental pressures in a prolonged and semi-immobile to immobile state as larvae and pupae (Rolff, Johnston and Reynolds, 2019). Furthermore, Bergmann's rule (1848) suggests a thermally adaptive association with body size in which larger body sizes are thought to be more advantageous in colder climates to buffer against the cold, which has

been observed in flour beetle species (Scharf, Sbilordo and Martin, 2014). Whereas, smaller body sizes allow for quicker heat loss to prevent overheating as observed in tropical butterfly *Hypolimnas bolina* (Kemp and Krockenberger, 2004).

However, when temperatures reach extremes, it can prove to be detrimental to insect survival. The mechanisms of cold tolerance and adaptations have been well-defined and documented into two categories: freeze avoidance and freeze tolerance (Storey and Storey, 1989). Freeze-avoidance strategies often include migration such as in monarch butterflies, and/or thermoregulatory behaviour such as basking (Masters, Malcolm and Brower, 1988) which are perhaps more likely and typical of tropical species, which while unlikely to freeze can be exposed to temperatures lower than thermal optimum.

We focus on a tropical species as thermal adaptation and tolerance studies have often favoured their temperate counterparts, which experience extremes at either end of the temperature spectrum (winter cold vs summer heat). Whereas, the perhaps slower and smaller changes in the tropics have been somewhat ignored (Slade and Ong, 2023). Yet, it is thought that the tropics themselves are the most vulnerable to climate change due to organisms already living close to their upper thermal limits (Deutsch *et al.*, 2008; Bonebrake and Deutsch, 2012) and the lack of seasonality in the tropics may hinder their ability to adapt to changing environments (Janzen, 1967).

Acclimation is a commonly observed phenomenon where, according to the beneficial acclimation hypothesis, previous exposure to less extreme temperatures provides a survival advantage during extreme exposure at a later stage by priming the organism (Leroi, Bennett and Lenski, 1994; Wilson and Franklin, 2002). Acclimation has been shown to occur in tropical systems such as in *Drosophila* (Kristensen *et al.*, 2008; Colinet *et al.*, 2013) and *Bicylus anynana* for both cold and heat tolerance in adults (Geister and

Fischer, 2007; Karl *et al.*, 2014). Therefore, further investigation within tropical species would have the potential to unveil pathways improving our understanding of heat and cold tolerances and whether they employ the same molecular mechanisms, and or whether the sensitivity of their triggers are similar to that of their temperate counterparts.

In heat acclimation, it is typically shown that the speed at which heat shock proteins (hsps) are triggered is hastened when responding to heat stress (Sejerkilde, Sørensen and Loeschcke, 2003). Hsps are stress response proteins that are highly conserved across eukaryotes (Karlin and Brocchieri, 1998). They are chaperones to prevent protein unfolding (denaturing) or help in refolding (Sørensen, Kristensen and Loeschcke, 2003), and are important in cell signalling and transduction. While not specific to thermal response, they are often the focus of thermal adaptation studies. For example, the hsp70 protein family in particular has been extensively studied in stress response but has also shown links to heat response and adaptation across insect species, including in tropical systems such as *Drosophila melanogaster* (Hartl, 1996) and brown planthopper *Nilaparvata lugens* (Huang *et al.*, 2017). However, aside from heat shock protein (hsp) studies, the physiological mechanisms that underlie heat tolerance and adaptations in insects remain poorly studied (González-Tokman *et al.*, 2020). The lack of non-hsp studies suggest there could perhaps be an evolutionary constraint with hsps being the only genes involved in thermal tolerance (González-Tokman *et al.*, 2020), but it is perhaps due to the lack of tropical studies that have led to this knowledge gap, as the production and maintenance of hsps are energetically expensive (Krebs and Loeschcke, 1994). Therefore, tropical species may have other mechanisms (that are currently understudied) to reduce mortality risks associated with high temperatures, which they are often exposed to (Fischer, Klockmann and Reim, 2014).

Physiological and molecular mechanisms underpinning cold tolerance have not been extensively studied in tropical models. Studies have otherwise mainly focused on phenotypic response in tolerance when exposed to low temperatures (Geister and Fischer, 2007; Karl *et al.*, 2014).

Heliconius butterflies have been extensively studied for their colour patterning and have a plethora of genetic resources (The *Heliconius* Genome Consortium 2012), but mechanisms that underpin their thermal adaptations have been relatively understudied. Here we use *Heliconius sara*, which is a particularly heat hardy species, found across the neotropics (Montejo-Kovacevich *et al.*, 2020), to investigate the effect of rearing temperature on development and thermal tolerance to both cold and heat extremes within a captive population. We hypothesize higher rearing temperatures will lead to 'typical' patterns in insect development such as decreased development time and reduced body sizes (Atkinson, 1994). With regard to thermal tolerance we hypothesize, that rearing temperature will lead to an acclimation affect enhancing thermal tolerance respectively to stress (cold vs hot).

Furthermore, through the use RNA sequencing, we aim to establish expression profiles associated with rearing temperature focusing on 'milestone' developmental stages of: 5th instar larvae, pupae and freshly eclosed adults, as well as adults exposed to either thermal extreme to determine the possible molecular underpinnings of acclimation and thermal tolerance throughout development. Through the combination of rearing and molecular techniques, we hope to bridge the gap between developmental and plastic phenotypes and the physiological mechanisms that underpin them, through a transcriptomic approach.

Methods

Rearing conditions

All specimens were reared from a captive, closely related, interbreeding stock of *H. sara.* Eggs were laid on *Passiflora auriculata* shoots, each egg clutch on a single shoot, was classified as a single 'batch'. Each batch was laid by multiple mothers, thus not all were siblings. For life history traits, 5 batches were analysed. There were 672 individuals in larval development time, and pupa weight, 674 for pupa mortality. There were 406 individuals for pupal development time and adult weight. For thermal tolerance traits, 3 batches were analysed for cold tolerance and, 5 batches were analysed for heat tolerance. There were 108 individuals cold tested and 159 were heat tested.

The eggs were hatched in standardised stock rearing conditions at 24°C, 75% relative humidity and a 12H:12H day:night cycle. Once hatched, the batch was split in half to be reared under one of the two strictly controlled thermal extremes, but field relevant temperatures of 19°C or 30°C (Montejo-Kovacevich *et al.*, 2020). Relative humidity and day:night cycle was kept the same as stock rearing conditions. Caterpillars were fed on *Passiflora biflora,* which was given ad libitum. Life history traits including, developmental time (to pupae and adult butterfly), pupal mortality, pupae weight, and adult weight were recorded for each individual to then be thermally tested.

Thermal Tolerance

Adult butterflies were randomly assigned to be tested for their tolerance against thermal extremes to either heat or cold, and researchers were blind to which rearing temperature butterflies originated from. Tests were assigned randomly and conducted 48 hours after eclosion from the pupa. All butterflies were syringe-fed artificial nectar solution twice,

once 24 hours after eclosion and once more on test day, where-after they were placed inside an envelope inside a blackout box for at least 30 minutes inside their rearing conditions before testing. After each thermal tolerance test was completed, the butterfly was killed and immediately preserved in RNAlater.

Cold tolerance was tested by exposing the butterflies to 3°C (±1°C) for 10 minutes before measuring the time taken for the butterfly to recover sufficiently from chill coma at 24°C, which was characterised by the ability to support itself on 3 or more legs. This was done by placing the butterfly inside a Pyrex glass beaker using forceps, with the top sealed with cling-film, submerged in cold water, inside a fridge. Once the cold exposure period was completed, the butterfly was then removed with forceps and placed inside a lidded recovery vessel. The recovery vessel was gently agitated with a few taps against the lid every 30 seconds to confirm if recovery had occurred.

Heat tolerance was tested as the time to heat coma (i.e heat knockout) which was characterised by the ability to no longer support itself on 3 or more legs, after exposure to 40°C (± 1°C). This was done by placing the butterfly inside a preheated Pyrex glass beaker with the top covered with cling film, inside a hot water bath and the beaker was periodically agitated to circulate heat and confirm heat coma (Montejo-Kovacevich *et al.*, 2020).

Analysis of life history and thermal tolerance

All analysis was completed using R Vers 4.0.0 (R core Team) and graphs were generated using 'ggplot' (Wickham, 2016).

The effect of rearing temperature on all trait variations was modelled with either Linear Mixed-Effect (LMM) or Generalised Linear Mixed-Effect (GLMM). Modelling depended

upon data distribution i.e if Gaussian, LMM were used, and if Gamma (e.g untransformed 'waiting time' variables) then GLMM. Within these models, temperature was treated as a categorical variable.

Sex (applicable only in adults) and 'Observer' (in thermal tolerance tests, which are subject to observer bias) were additional explanatory factors considered in the models. In addition, because a variable number of offspring of the same egg clutch (or 'Batch') were reared, and there may be differences between these, we included 'Batch' as a random effect in the model. For heat tolerance tests, there were individuals that did not knock out within two hours of testing; they were included in analysis with knock out times of 120 minutes.

The modelling protocol for each trait was the same for every trait, in which a list of nested candidate models, combining all explanatory variables plus any pairwise statistical interaction, was first generated. These models were then fit to the data and ranked according to their AICc scores (Burnham and Anderson, 2004). The model with the lowest AICc value with the fewest explanatory variables, and at least 2 AICc lower than the 'Null' model (which only contained the random factor and no other explanatory variable), was selected. If the selected model contained temperature, then the selected model was then used to estimate the effect size of temperature. The model fitting was carried out using the R package lme4 (Bates *et al.*, 2015), and model selection with AICcmodavg (Mazerolle, 2023).

The effect size of temperature was calculated as the ratio between the estimated marginal means (as predicted by the selected models) for individuals in the corresponding temperature. These effect sizes were estimated using the package emmeans (Searle, Speed and Milliken, 2023b).

RNA Extraction

Sample sizes used for RNA sequencing for each rearing condition, developmental stage, and thermally tested condition can be found in Table 1, and all samples were female to avoid confounding effects of sex. Samples used for the post-testing conditions were chosen with recovery (for cold test) and knockout (for heat test) times that were within minutes (3.5-5 for cold recovery, and 6-15 minutes in heat KO times) between one another, across rearing temperatures where possible, for standardisation. Heat-tested adults that did not knock-out after 2 hours of heat testing were also included as an additional treatment category. Pupae were preserved as soon as the pupal casing had hardened, and 5th instar caterpillars were preserved post-moult, as soon as their spines were pigmented and hardened, to standardise the developmental stage. Samples of larvae and pupae were also included from the "stock" population, which is kept at 24°C to provide an additional comparison point and to increase the sample sizes of these stages.

Rearing temperature	**Adult control**	**cold test**	**heat test KO (heat coma)**	**heat test (no heat coma)**	**5th instar caterpillar**	**Pupae**
30°C	5	5	5	4	5	3
19°C	5	3	5	5	3	4
24°C ('stock')	0	0	0	0	4	4

Table 1: Sample size numbers of each rearing condition and each developmental stage, in each thermal test.

RNA extraction was conducted using the QIAGEN RNAeasy mini kits following the manufacturer's protocol, including the additional QIAGEN DNAase step to remove any additional DNA contamination (QIAGEN, no date b, no date a). RNA concentration and quality was assessed with nanodrop (260/280 and 260:230 > 1.8), Qubit Fluorometer (Thermo Fisher Scientific), and the Agilent RNA 6000 nano kit procedures (Aligent) (total

concentration (ng) > 20 - 1000 ng in 10-50 µl). All extracted RNA samples were stored at -80°C plated in the 96 well plate and shipped with dry ice.

Sequencing

Library preparation, sequencing, trimming, and quality control were conducted by the Liverpool Centre for Genomic Research. Library preparation was completed using NEBNext polyA selection and Ultra II directional RNA library preparation kits. Paired-end (2x150 bp) sequencing was conducted with one lane illumina NovaSeq using S4 chemistry. Raw Fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1. The option -O 3 was used, so the 3' end of any reads which match the adapter sequence for 3 bp or more were trimmed. The reads were then further trimmed using Sickle version 1.200 with a minimum window quality score of 20. Reads shorter than 15 bp after trimming were removed. Multiqc was run to check the quality of sequences generated.

Sequence alignment

HISAT2 was used to align sequences against the *H. sara* genome (*NCBI*). Stringtie was used to assemble and quantify transcripts, with the stringtie 'merge' option to allow novel genes to be included against the existing genome annotation. Packages "IsoformSwitchAnalyzeR" and "stringr" were used to retrieve the gene names and was conducted in R Vers 4.0.0 within Rstudio (R core team).

Differential expression

To find which genes were significantly differentially expressed (DE) between our different rearing conditions and tested individuals, we used the Bioconductor package 'EdgeR' (Robinson, McCarthy and Smyth, 2009). Following the EdgeR manual, filtering

and normalisation were conducted within the Generalized linear model framework, with a single model design for each developmental stage. Models for larvae and pupae only contained rearing temperature as a factor, while adults also contained thermal tolerance test type (or control) as a second factor (Chen, Y., McCarthy, D., Ritchie, M., Robinson, M., Smyth, G., & Hall, 2020). These models were also used to calculate the Biological coefficient of variation (BCV) between samples.

Gene set enrichment analysis

A gene set enrichment analysis (GSEA) was conducted to determine if any functional category of genes were significantly enriched in any of the sets of DE genes identified. First, to identify the functional categories that genes belonged to, a BLASTx using the *H. sara* protein sequences for the entire RNAseq gene count matrix, was run against the UniProt *Drosophila melongaster* proteome (UniProt), with an e-value of <0.01 for homology level hits. Once gene names of the *D. melanogaster* equivalent were obtained, DE gene lists were run through DAVID (Database for Annotation, Visualization and Integrated Discovery) an online bioinformatics resource that provides functional annotation tools to find enriched GO (gene ontology) terms (Sherman *et al.*, 2022). DAVID uses a modified Fischer exact test (EASE score) to determine enriched GO terms (Sherman *et al.*, 2022). Only GO terms with EASE scores <0.05 and Benjamini scores of <0.05 were considered to be significant. The latter accounted for multiple-testing, giving corrected false discovery rates. All gene lists were run through DAVID against a background gene list containing all the genes in the original *H. sara* gene count matrix that had a BLASTx match against *D. melanogaster*, to act as our 'genome'.

With BLASTx we also searched for genes homologous to heat shock proteins (hsps) that were DE between the contrasts. This is because hsps are well documented to be

associated with temperature response (Feder and Hofmann, 1999; Krebs and Bettencourt, 1999; Sørensen, Kristensen and Loeschcke, 2003).

Results

Life history

The effect of temperature on development time was substantial, it took half the time at 30°C (Figure 1) for larvae (30°C:19°C, effect size = 0.45x ± 0.01) and pupae to develop (30°C:19°C, effect size = 0.43x ± 0.01). The strongest ranked model for larval development time (AICcWt = 1) contained temperature only, while pupal development time contained both temperature and sex (AICcWt = 0.71), where males tended to develop quicker than females. Sex could not be accounted for in larval development time, as sex could not be determined until after eclosion and only about 60% survived to adulthood.

For body size, hotter rearing temperatures had inverse effects on pupa and adult weights. In pupae, hotter temperatures led to substantially smaller pupae (30°C:19°C, effect size = 0.79x ± 0.09) (Figure 1). In pupa weight, the highest-ranked model also contained development time, which was also found to interact with temperature (AICcWt = 0.83). Pupa weight generally decreased with longer development time, but the interaction with temperature revealed there was little effect of development time at 30°C while at 19°C longer development times led to substantially smaller pupa (Supplementary Figure 1). In adults, hotter temperatures lead to larger adults (30°C:19°C, effect size = 1.56x ± 0.4) (Figure 1). For adult weight, the highest ranked model included pupal development time interacting with temperature, with the addition of sex, where females tended to be larger than males (AICcWt = 0.6). Longer development times showed a negative trend with adult weight. However, the interaction between pupal development time and temperature

revealed that longer pupal development at 30°C led to larger adults, while at 19°C appeared to have no clear correlation with adult weight (Supplementary Figure 2).

Rearing temperature had no effect on pupa mortality, with the null model being the preferred model (AICcWt = 0.73) (Figure 1).

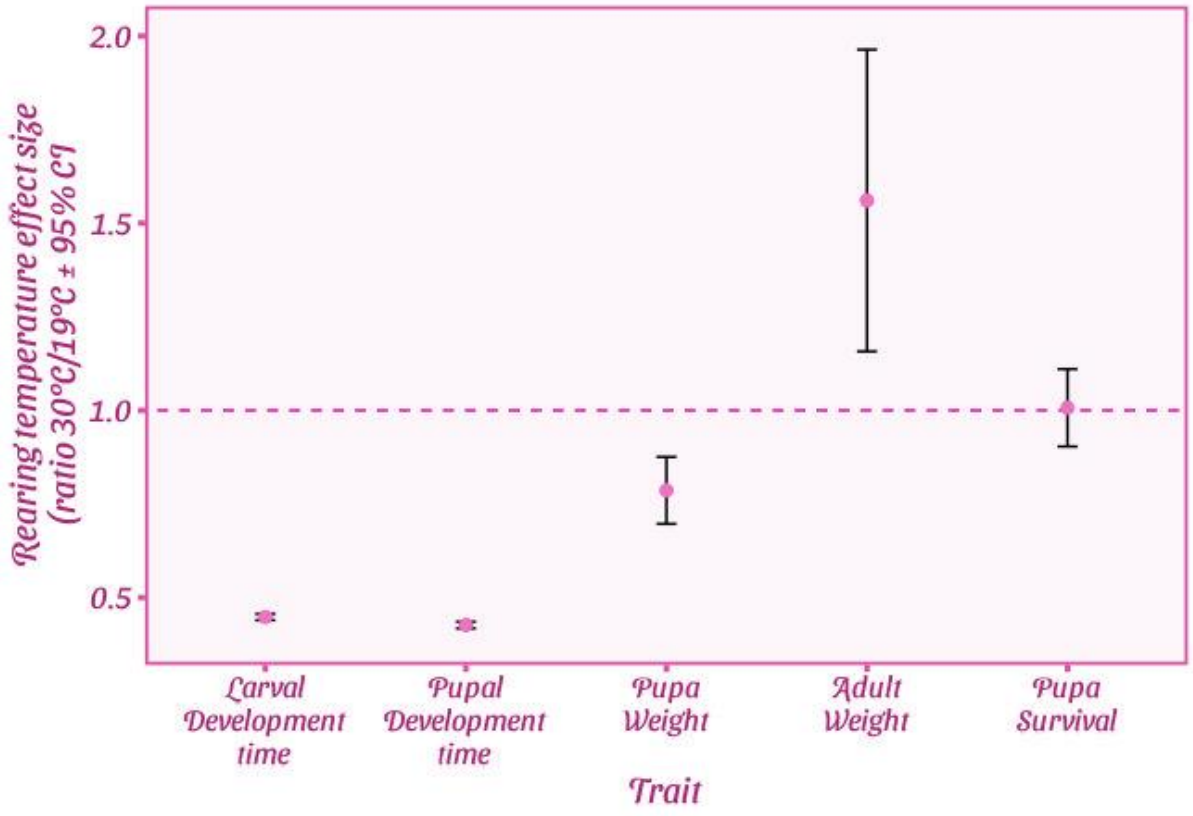


Figure 1: The effect of increased rearing temperature on developmental traits as the ratio between the estimated marginal means predicted by the selected models at 30°C/19°C with ± 95% confidence intervals

Thermal tolerance

For both cold tolerance and heat tolerance, the highest ranking models contained rearing temperature as explanatory factors. In cold tolerance was there a substantial effect, where higher rearing temperatures increased the time it took for individuals to recover from chill coma by 1.34x ± 0.14 suggesting lower cold tolerance (Figure 2). The highest

ranking model also contained body size, sex (with an interaction to temperature) and observer, but this model (AICcWt = 0.45) was not 2 AICc units smaller than the model with just temperature and observer alone (AICcWt = 0.24). This suggests that colder rearing temperatures increase cold tolerance.

In heat tolerance, the highest ranking model contained rearing temperature and observer (AICcWt= 0.41). Temperature had a substantial effect on time to heat knock coma where higher rearing temperatures increased time to heat coma by 1.86 ± 0.78 suggesting greater heat tolerance (Figure 2).

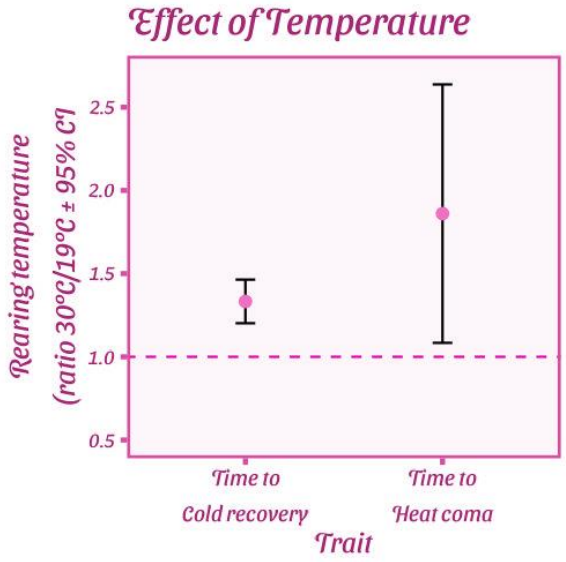


Figure 2: The effect of increased rearing temperature on thermal tolerance traits as the ratio between the estimated marginal means predicted by the selected models at 30°C/19°C with ± 95% confidence intervals

Differential expression and Gene set Enrichment Analysis

The biological coefficient of variation (BCV) for adult samples was 0.63, for larvae it was 0.61 and for pupae it was 0.60. This indicated that the natural variation in gene

expression between RNA-seq samples was relatively moderate, and suggests a consistent level of biological diversity within the dataset (See supplementary figures 3-5).

Larvae

As shown in Figure 3, in overall gene expression, larvae tend to cluster together by their rearing temperature, with clear separation on dimension 2, which explained up to 16% of differences between individuals, and those reared at 24°C also separate away from the other two rearing temperatures on dimension 1 which explained 28% of variation. This was then reflected in the numbers of differentially expressed (DE) genes, where the greater rearing temperature differences yielded more DE genes. The contrast between 30°C and 19°C had the most DE genes (639 up- and 546 down-regulated genes), then 30°C and 24°C (294 up- and 540 down-regulated genes), while the contrast between 24°C and 19°C had the fewest DE genes (229 up- and 264 down-regulated genes) (Table 2). This cascade was also reflected in the number of significantly enriched GO terms, which is unsurprising as the more DE genes there are, there are typically greater number of enriched GO terms (Table 2). GO terms were broad without particular specificity (Table 2).

Although, greater temperature differences led to a larger number of DE expressed genes, there was little overlap of genes between the contrasts with only 4 genes (2 up- and 2 down-regulated) shared between all three contrasts. While the pairs of contrasts had less than a fifth of genes shared, with "30°C compared to 19°C" and "24°C compared to 19°C", sharing 116 up-regulated genes and 116 down-regulated genes in common; "30°C compared to 19°C" and "30°C compared to 24°C" having 66 up-regulated genes and 197 down-regulated genes in common; and "30°C compared to 24°C" and "24°C and 19°C" had no other genes in common. (Figure 4). Therefore, while greater temperature differences

led to greater DE, as the genes largely did not overlap, it suggests each temperature has its own unique temperature response mechanisms.

Within the genes up-regulated at 30°C, there was one homologue to a *D. melanogaster* heat shock protein gene, (Hsc70-4) when compared to 24°C. This gene, in addition to *D. melanogaster* equivalent to Hsp23 was also up-regulated at 30°C when compared to 19°C.

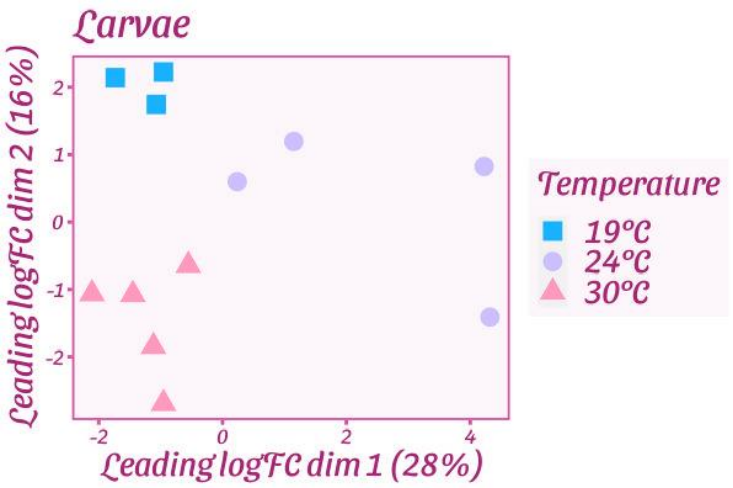


Figure 3: Multidimensional-scaling of larvae samples by overall gene expression, showing separation by rearing temperature (denoted by colour and shape) but clustering within the same rearing temperature.

Contrast	Up-reg	Down-reg	Up-reg GO terms	Down-reg GO terms
30°C vs 19°C	639	546	27 GO:0002181~cytoplasmic translation (BP) GO:0022626~cytosolic ribosome (CC) GO:0031057~negative regulation of histone modification (BP) GO:0005840~ribosome (CC) GO:0003690~double-stranded DNA binding (MF) GO:0003735~structural constituent of ribosome (MF)	32 GO:0006333~chromatin assembly or disassembly (BP) GO:0044877~macromolecular complex binding (MF) GO:0012505~endomembrane system (CC) GO:0040003~chitin-based cuticle development (BP) GO:0005783~endoplasmic reticulum (CC) GO:0030527~structural constituent of chromatin (MF) GO:0006334~nucleosome assembly (BP)
30°C vs 24°C	294	560	8 GO:0033181~plasma membrane proton-transporting V-type ATPase complex (CC) GO:0046961~proton-transporting ATPase activity, rotational mechanism (MF) GO:1902600~hydrogen ion transmembrane transport (BP) GO:0033179~proton-transporting V-type ATPase, V0 domain (CC) GO:0000220~vacuolar proton-transporting V-type ATPase, V0 domain (CC) GO:0000221~vacuolar proton-transporting V-type ATPase, V1 domain (CC)	15 GO:0040003~chitin-based cuticle development (BP) GO:0008010~structural constituent of chitin-based larval cuticle (MF) GO:0005576~extracellular region (CC) GO:0008061~chitin binding (MF) GO:0005615~extracellular space (CC) GO:0006032~chitin catabolic process (BP)

24°C vs 19°C	229	264	3 GO:0008010~structural constituent of chitin-based larval cuticle (MF) GO:0031012~extracellular matrix (CC) GO:0040003~chitin-based cuticle development (BP)	36 GO:0012505~endomembrane system (CC) GO:0005783~endoplasmic reticulum (CC) GO:0006888~ER to Golgi vesicle-mediated transport (BP) GO:0046961~proton-transporting ATPase activity, rotational mechanism (MF) GO:1902600~hydrogen ion transmembrane transport (BP) GO:0038024~cargo receptor activity (MF)

Table 2: numbers of significantly DE expressed genes and related GO terms between larval contrasts. Up- and down-regulated numbers denote expression direction of genes for the contrast factor on the left versus the factor on the right. The top two GO terms per GO term category were also included when possible (otherwise, all were presented if there were <6 total) for each Up- and Down-regulated gene lists with the lowest Benjamini score <0.05. MF = molecular function, CC - cellular component, BP = Biological process. See SI for all other enriched GO terms not in this table

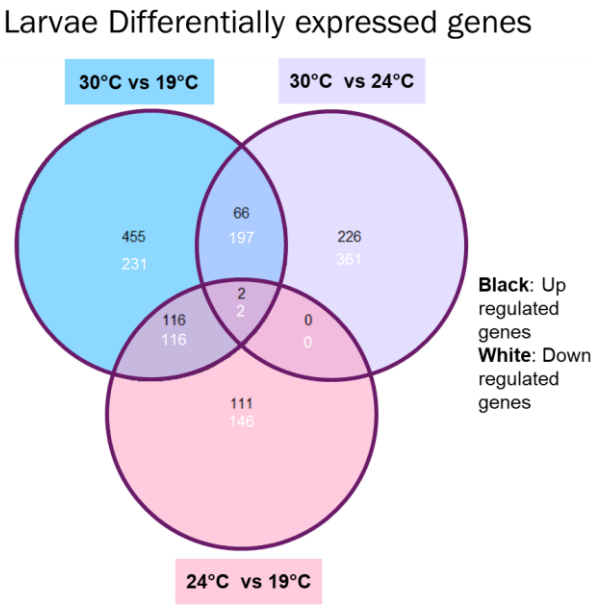


Figure 4: Numbers of significantly differentially expressed genes between three contrasts in larval individuals: 30°C vs 19°C (blue), 30°C vs 24°C (in purple) and 24°C vs 19°C. Also showing the numbers of the same overlapping genes between the contrasts. In black numbers are the numbers of significantly up-regulated genes, and white numbers are the numbers of significantly-down regulated genes for the left rearing temperature of each contrast and vice versa for the temperature on the right side of each contrast.

Pupae

We see in (Figure 5) that the overall gene expression of pupae reared at 30°C and 24°C clustered together and somewhat away from those reared at 19°C via dimension 2, which explains up to 14% of differences between individuals. Oddly enough, this was not reflected in their DE, which rather followed similar patterns to larvae where greater rearing temperature differences yielded more DE genes. The contrast between 30°C and 19°C had the most DE genes (225 up- and 313 down-regulated), then 30°C and 24°C (49 up- and 119 down-regulated) while the contrast 24°C and 19°C had no significantly DE genes whatsoever (Table 3). The larger number of DE genes once again also led to more significantly enriched GO terms, the GO terms similarly to larvae were broad involving

up-stream mechanisms (Table 3). However, the go term "chitin-based cuticle" was significantly down-regulated GO terms in individuals reared at 30°C when compared to individuals reared at both 24°C and 19°C which may due to slight differences in development during preservation (Table 3).

Although, unlike larvae, the significantly DE genes between contrasts involving individuals reared at 30°C do overlap, as 128 genes (31 up- and 97 down-regulated), which was over 75% of the DE genes between contrast 30°C and 24°C, were also found in the contrast between 30°C and 19°C (Figure 6). The increase in DE with increasing rearing temperature differences, and overlap of genes from overlapping temperatures, suggests a cascade effect with temperature in pupa response. Within the up-regulated genes in individuals reared at 30°C in comparison to 19°C, there were two genes with *D. melanogaster* equivalents that encoded heat shock proteins, Hsc70-4 and Hsp23. Hsp23 was also significantly up-regulated when 30°C was compared to 24°C, but not Hsc70-4.

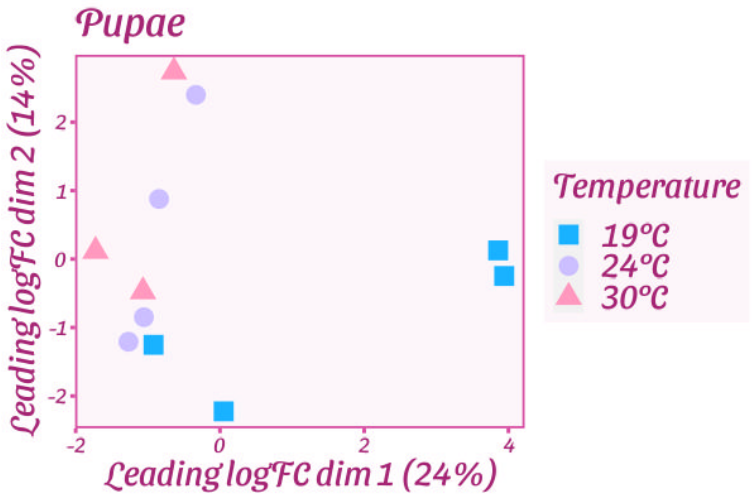


Figure 5: Multidimensional-scaling of pupae samples by overall gene expression, showing some separation by rearing temperature (denoted by colour and shape) with some clustering within the same rearing temperature.

Contrast	Up-reg	Down-reg	Up-reg GO Terms	Down-reg GO Terms
30°C vs 19°C	225	313	16 GO:0031057~negative regulation of histone modification (BP) GO:0016584~nucleosome positioning (BP) GO:0003690~double-stranded DNA binding (MF) GO:0031490~chromatin DNA binding (MF) GO:0000786~nucleosome (CC) GO:0000785~chromatin (CC)	10 GO:0040003~chitin-based cuticle development (BP) GO:0055085~transmembrane transport (BP) GO:0005615~extracellular space (CC) GO:0022857~transmembrane transporter activity (MF) GO:0008010~structural constituent of chitin-based larval cuticle (MF) GO:0016021~integral component of membrane (CC)
30°C vs 24°C	49	119	None	6 GO:0040003~chitin-based cuticle development (BP) GO:0005615~extracellular space (CC) GO:0008010~structural constituent of chitin-based larval cuticle (MF) GO:0046692~sperm competition (BP) GO:0032504~multicellular organism reproduction (BP) GO:0031012~extracellular matrix (CC)
19°C vs 24°C	0	0	N/A	N/A

Table 3: Numbers of significantly DE expressed genes and related GO terms between pupa contrasts. Up- and down-regulated numbers denote expression direction of genes for the contrast factor on the left versus the factor on the right. The top two GO terms per GO term category were also included when possible (otherwise, all were presented if there were <6 total) for each Up- and Down-regulated gene

lists with the lowest Benjamini score <0.05. MF = molecular function, CC - cellular component, BP = Biological process. See SI for all other enriched GO terms not in this table

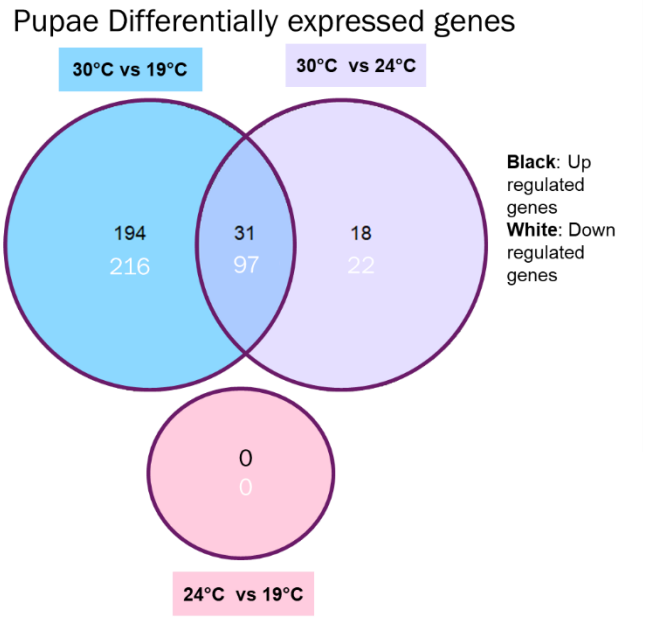


Figure 6: Numbers of significantly differentially expressed genes between three contrasts in pupal individuals: 30°C vs 19°C (blue), 30°C vs 24°C (in purple) and 24°C vs 19°C (pink). Also showing the numbers of the same overlapping genes between the contrasts. In black, are the numbers of significantly up-regulated genes, and in white are the numbers of significantly down-regulated genes, for the left rearing temperature of each contrast, and vice versa for the temperature on the right side of each contrast.

Adult Butterflies

The multi-dimension scaling (MDS) plot of overall gene expression (Figure 7) shows adults generally clustering by rearing temperature on dimension 1 which explains 11% of differences between individuals. However, there does also appear to be tighter clustering among the individuals that were heat stressed but did not enter heat coma after 2 hours (hereafter referred to as non-heat-KO) within their rearing temperatures, compared to all other individuals. This was then reflected in their DE, as when rearing temperature alone was compared (30°C vs 19°C), there was the greatest number of

significantly DE genes (2901 up- and 2267 down-regulated (Table 4)). When comparing thermal tolerance testing, there were no DE genes between cold-tested individuals and their controls regardless of rearing temperature. There were also no DE genes between individuals that were exposed to heat stress and did knock out after a few minutes (hereafter referred to as heat KO) and the controls. However, when non-heat-KOs were compared to their controls, and KO counterparts, there were more DE between individuals reared at 19°C than 30°C. Within individuals reared at 19°C there were 76 up- and 8 down-regulated genes when compared to the controls and 83 up- and 13 down-regulated genes when compared to KOs (Table 4)). When reared at 30°C, non-heat-knockouts had 53 up-regulated genes and 7 down-regulated genes compared to controls, and 42 up-regulated genes and 9 down-regulated genes when compared to KO individuals (Table 4)). When comparing the DE genes between all 4 contrasts involving non-heat-KOs, all overlapping genes had been expressed in the same direction with an overlap of 16 significantly upregulated genes but otherwise few to no overlap between other contrasts (Figure 8)). Within these 16 overlapping significantly up-regulated genes in the non-heat-KO individuals, contained three genes that had *D. melanogaster* equivalents that encode heat shock proteins (Hsp68, Hsp83 and Hsc70-4) known to be directly involved in heat response (Xiao and Lis, 1989; McColl, Hoffmann and McKechnie, 1996). Furthermore, despite there being distinct sets of DE genes in the no-heat-KO individuals reared at each temperature, we found no genes with expression patterns showing a significant interaction between rearing temperature and knock-out treatment (Table 4)).

The gene enrichment on the up-regulated genes in non-heat-KO individuals compared to controls and heat-KO individuals across rearing temperatures yielded significantly enriched GO terms that overlapped and were specific to heat response (Table 4).

Comparison between developmental stages

Between all three developmental stages (adult, pupae, and larvae) when comparing the DE genes of those reared at 30°C to 19°C, there is relatively little overlap. Between all three stages there were 63 genes that were DE where 55 were expressed in the same direction (31 up- and 24 down-regulated). There was also 8 DE genes that were expressed differently depending on rearing developmental stage: 4 up- and 1 down-regulated in adults but were reversed in the pupae and larvae; 2 down-regulated in pupae which were otherwise up-regulated in the other stages; and 1 down-regulated in larvae that were up-regulated in the other developmental stages (Figure 9)). This lack of overlap continues between the pairs of developmental stages, with pupae having the least overlap with both adults (59 up- and 75 down-regulated) and larvae (12 up- and 30 down-regulated) (Figure 9)). However, adults and larvae do appear to have a somewhat substantial number of shared genes, with an additional 174 up- and 120 down-regulated genes shared (Figure 9)). When reared at 30°C in all three developmental stages, two up-regulated genes correspond to *Drosophila* heat shock proteins (Hsp68, Hsc70-4). In adults, an additional hsp gene, Hsp83, was significantly up-regulated when reared at higher temperatures.

Even though there was not a large number of overlapping genes, they did have overlapping enriched GO terms (Table 2-4) such as “chromatin”, “heterochromatin assembly” and “ribosome” etc. (see supplementary information for enriched GO terms that are not stated in the main tables)

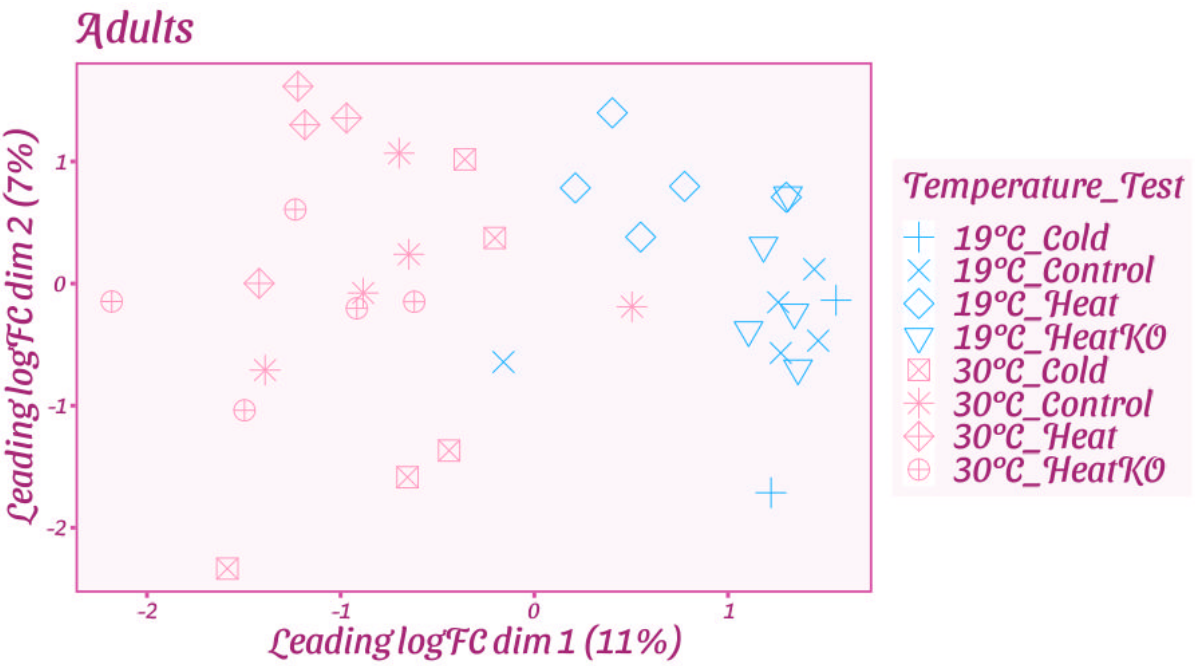


Figure 7: Multidimensional-scaling of adult samples by overall gene expression, showing separation by rearing temperature (blue and pink for 19°C and 30°C degrees respectively), and minor separation within each chamber by stressor groups (symbols denote each combination of rearing temperature and stress treatment, heat_KO being the individuals that were exposed to heat stress and fell into heat coma).

Rearing Temperature	Contrast	Up-reg	Down-reg	Up-reg GO Terms	Down-reg GO Terms
Both	30°C vs 19°C	2901	2267	20 GO:0005730~nucleolus (CC) GO:0003676~nucleic acid binding (MF) GO:0031057~negative regulation of histone modification (BP) GO:0031490~chromatin DNA binding (MF) GO:0006364~rRNA processing (BP) GO:0005737~cytoplasm (CC)	34 GO:0005739~mitochondrion (CC) GO:0003735~structural constituent of ribosome (MF) GO:0005840~ribosome (CC) GO:0006412~translation (BP) GO:0002181~cytoplasmic translation (BP) GO:0016491~oxidoreductase activity (MF)
19°C	Heat test: no KO vs control	76	8	13 GO:0051082~unfolded protein binding (MF) GO:0006457~protein folding (BP) GO:0042026~protein refolding (BP) GO:0051087~chaperone binding (MF) GO:0034663~endoplasmic reticulum chaperone complex (CC)	None
	Heat test: no KO vs KO	83	13	9 GO:0051082~unfolded protein binding (MF) GO:0042026~protein refolding (BP) GO:0006457~protein folding (BP) GO:0031072~heat shock protein binding (MF)	None

	Heat test: KO vs control	0	0	N/A	N/A
	Cold Test vs Control	0	0	N/A	N/A
30°C	Heat test: no KO vs control	53	7	14 GO:0042026~protein refolding (BP) GO:0051082~unfolded protein binding (MF) GO:0031072~heat shock protein binding (MF) GO:0006457~protein folding (BP)	None
	Heat test: no KO vs KO	42	9	12 GO:0051087~chaperone binding (MF) GO:0051082~unfolded protein binding (MF) GO:0042026~protein refolding (BP) GO:0006457~protein folding (BP)	None
	Heat test: KO vs control	0	0	N/A	N/A
	Cold Test vs Control	0	0	N/A	N/A
Both: Temperature*Test 30°C vs 19°C	Heat test: no KO vs control	0	0	N/A	N/A
	Heat test: no KO vs KO	0	0	N/A	N/A

	Heat test: KO vs control	0	0	N/A	N/A
	Cold Test vs Control	0	0	N/A	N/A

Table 4: Number of significantly differentiated expressed genes for each adult contrasts. With the * denoting interaction. Up- and down-regulated numbers denote expression direction of genes for the contrast factor on the left versus the factor on the right. The top two GO terms per GO term category were also included when possible (otherwise, all were presented if there were <6 total) for each Up- and Down-regulated gene lists with the lowest Benjamini score <0.05. MF = molecular function, CC - cellular component, BP = Biological process. See SI for all other enriched GO terms not in this table.

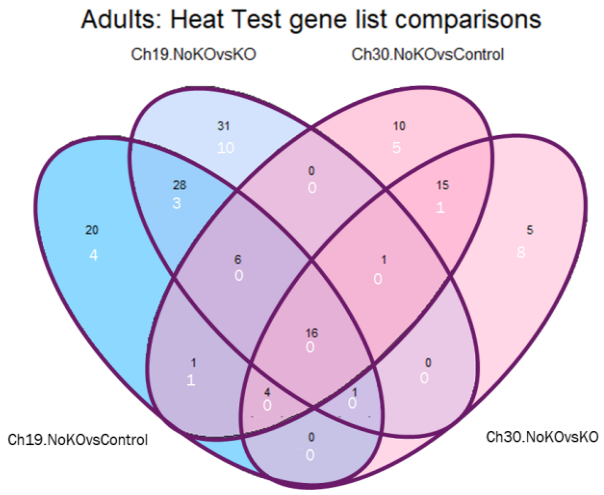


Figure 8: Numbers of significantly DE genes and their overlaps between contrasts involving non-heat-KO against their control and knockout counterparts reared at 19°C (blues) and 30°C (pinks). Numbers of significantly up-regulated genes are in black, and numbers of significantly down-regulated genes are white numbers, for the non-knockouts versus controls or knock-outs.

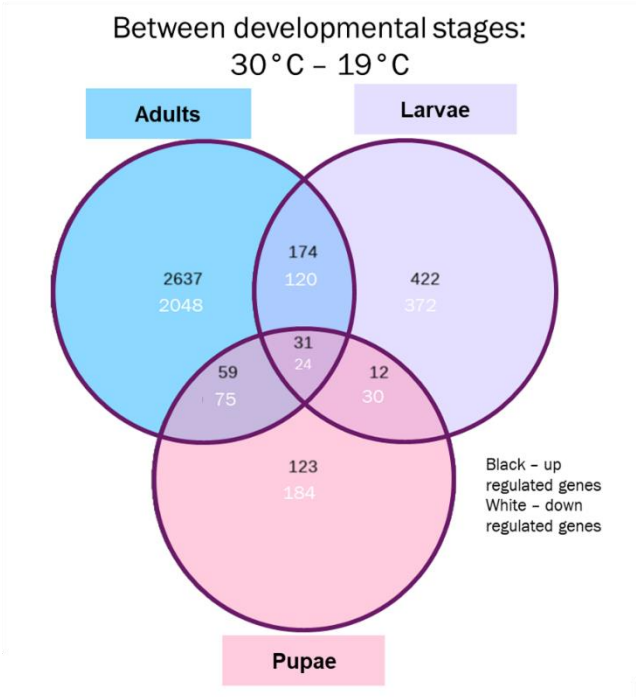


Figure 9: Number of significantly DE genes in rearing temperature contrast 30°C vs 19°C between three developmental stages of adult (blue), larvae (purple) and pupae (pink), and the overlaps between them. Numbers of significantly up-regulated genes are in black, and numbers of significantly down-regulated genes are in white for individuals reared at 30°C compared to 19°C (and vice versa for 19°C compared to30°C).

Discussion

Temperature effects on developmental traits

It was unsurprising that the developmental time in *H. sara* halved when reared at 30°C in comparison to 19°C, as warmer (non-lethal) temperatures have consistently been shown to decrease developmental time across insects, in both temperate (Fischer and Karl, 2010) and tropical species (Steigenga and Fischer, 2009), due to higher temperatures speeding up metabolism (Nedvěd, 2009). For body size in pupae, there did appear to be a trend which followed the temperature-body-size rule, a common reaction norm, where warmer temperatures lead to smaller body sizes (Atkinson, 1994), but surprisingly this was reversed in adult weights, where warmer rearing temperatures ultimately led to larger adult butterflies. Furthermore, our findings showed an interesting interaction between temperature and development time. Typically, longer development times lead to larger body sizes, but when individuals were reared at 19°C a reverse trend was observed where longer development times led to smaller pupae. This could be associated with optimal developmental temperatures (Kingsolver *et al.*, 2007; Steigenga and Fischer, 2009). While 19°C and 30°C were field-relevant temperatures (Montejo-Kovacevich *et al.*, 2020) they are on the extreme ends, and perhaps the colder 19°C was inherently more stressful, leading to longer developmental periods and smaller body sizes (Chown and Gaston,

2010). Longer pupal development time at 30°C also led to larger adults but this was likely because larger pupae tend to take longer to develop.

Temperature effects on thermal tolerance in adult butterflies

In cold and heat tolerance testing, we observed the expected effects of acclimation where colder rearing temperatures increased cold tolerance (with shorter cold recovery times) and warmer rearing temperatures increased heat tolerance (with longer times to heat coma). This is a common pattern across species observed in many acclimation studies including several *Drosophila* species (Kristensen *et al.*, 2008; Nyamukondiwa *et al.*, 2011) and lepidopteran species such as *Bicyclus anynana* (Fischer *et al.*, 2010; Karl *et al.*, 2014).

Differential expression and Gene set enrichment analysis

Across developmental stages

In both larvae and pupae, the larger the temperature differences led to a greater number of differentially expressed (DE) genes. While in pupae the genes did appear to subset suggesting a cascading effect with temperature, this was not the case in larvae, suggesting that each rearing temperature could be triggering its own specific set of genes. This could suggest that larvae have more or different mechanisms when responding to temperature. Differential temperature responses by developmental stage have been observed previously in flesh fly, *Sarcophaga similis* (Harada and Goto, 2017), and in fruit fly *Bactrocera tau* (Huang *et al.*, 2020) and in *D. melanogaster* (Austin and Moehring, 2019). Temperature response has many mechanisms that often work in conjunction with each other (Mutamiswa *et al.*, 2023). As larvae are in a semi-mobile life stage, they may employ different behavioural mechanisms that may adjust their gene expression at each rearing temperature (Briscoe *et al.*, 2012), whereas in pupae due to their immobility, they may rely solely on a single mechanism for example heat shock proteins. The additional up-

and down-regulated genes in pupae reared at 30°C may aid in pupal survival at higher temperatures. This was supported by our GSEA where we found a significantly enriched GO term for "protein refolding" in the up-regulated genes in pupae at 30°C when compared to 19°C, as higher temperatures can affect protein structure (denaturation) (Lee *et al.*, 2019).

Between the three developmental stages, when comparing the two extreme rearing temperatures, both larvae and adults had a greater number of up-regulated genes than down at higher rearing temperatures, but this was reversed for pupae. This could be because the pupal stage of a butterfly is the most transcriptionally active stage (Ozerova and Gelfand, 2022) therefore, an increase in temperatures which hastens metabolic rate may need to be slowed down (i.e down-regulated) to not cause timing issues within the metamorphosis process. In contrast to larvae and adults where the priority could be thermal response, which was reflected in the greater number of shared genes between larvae and adults in comparison to pupae. This could be supported by evidence found in fruit fly *Bactrocera tau* where their pupae appeared to be the least temperature-sensitive stage (Huang *et al.*, 2020) and in *D. melanogaster* where all life stages except the pupa stage showed local adaptation to temperature (Austin and Moehring, 2019).

Our GSEA on the DE genes across all developmental stages when comparing the most extreme rearing temperatures did not show specificity in enriched GO terms. Rather, instead were broad terms involved with DNA binding, chromatin structure etc. (except for one GO term in pupae) suggesting perhaps higher rearing temperatures changes how genes are expressed in up-stream mechanisms rather than specific downstream pathways. While, chromatin modifications in association with methylation have been linked to heat acclimation in animals as a general (Tetievsky and Horowitz, 2010; Wu,

Zhang and Li, 2020), this has been understudied in insects, aside from a possible suggestion in *D. melanogaster*, (Colinet *et al.*, 2013). These mechanisms did appear to be up-regulated, in individuals reared at higher temperatures, but there did not appear to be an association with heat acclimation in *H. sara* as we had not observed an effect of heat acclimation on thermal tolerance. In our GSEA on significantly down-regulated gene lists (which would be up-regulated in our cold-reared individuals), while there were more enriched GO terms, there was again little specificity. There were some GO terms associated with chitin production, but this could be due to slight developmental timing differences between the two rearing temperatures, as those at colder temperatures develop slower and so may still be producing cuticle post-moult, while at warmer temperatures individuals have passed this stage.

The lack of DE genes between controls and thermally tested individuals (cold tested and heat KOs) is perhaps due to a thermal exposure period that was not long enough to elicit a response on a transcriptomic level, compared to other experiments that had hours-long exposure and recovery periods to elicit such response (Teets *et al.*, 2012). However, as mentioned earlier, we did show phenotypic evidence of a cold acclimation effect with rearing temperature on cold tolerance, therefore perhaps the broad enriched GO terms elude that cold acclimation is complex and involves up-stream non-specific or perhaps undefined mechanisms and pathways.

In thermally tested adults

When comparing the DE within adults, only heat-tested individuals that did not fall into heat coma showed any DE when compared to their control/knockout counterparts within rearing temperatures. There were overall fewer DE genes in this comparison when reared at 30°C versus 19°C, which could suggest a reduced reaction to heat stress at higher rearing temperatures (Karl *et al.*, 2012). However, this difference was negligible

and no genes were identified when specifically testing for the interaction between rearing temperature and non-knockout heat status. Furthermore, in the GSEA for these up-regulated DE genes, all gene lists showed specific enriched GO terms associated with heat stress and adaptation. While we were unable to establish a link between heat tolerance and rearing temperature this suggests that these non-heat-KO individuals have unique traits that allow them to withstand extreme heat for long periods of them. These adaptations could be associated with the speed and or concentration of heat shock protein (hsp) (Sejerkilde, Sørensen and Loeschcke, 2003) expression as all non-heat-KO individuals had the same significant up-regulation of Hsp70 chaperones (Hsp68 and Hsc70-4) and Hsp90 chaperones (Hsp83). In *Drosophila* these genes are tightly linked to thermal adaptation and heat response– functioning in protein folding and refolding(Hartl, 1996; McColl, Hoffmann and McKechnie, 1996; Boher *et al.*, 2012; Gu *et al.*, 2021) i.e mediating protein denaturing which occurs as high heat (Hartl, 1996; Lee *et al.*, 2019). Furthermore, in temperate butterfly *Lycaena tityrus*, higher concentrations of hsp70 expression have been associated with increased heat tolerance (Karl *et al.*, 2009).

Conclusions

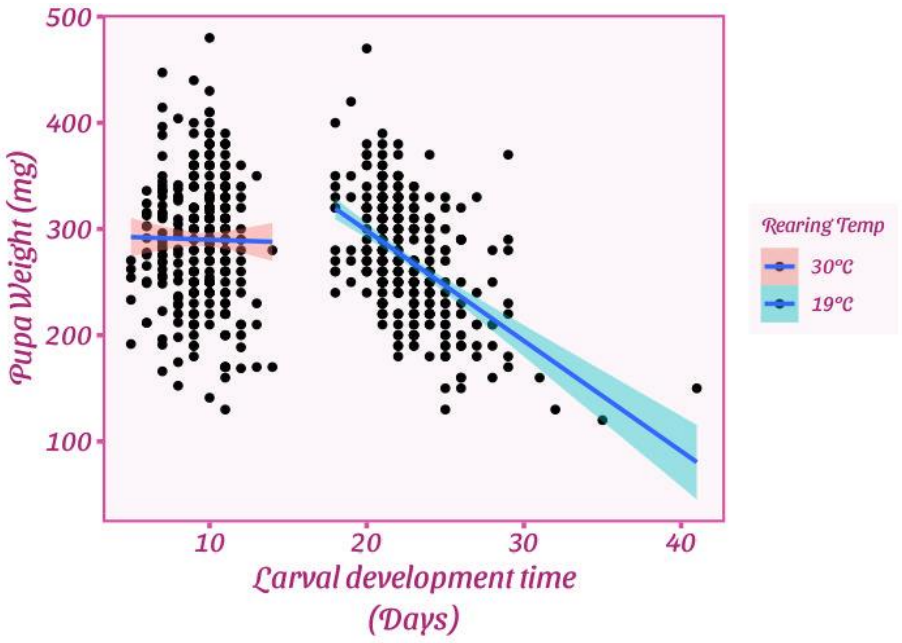
In conclusion, we observed predictable shifts in developmental time and body size (with pupae) in response to temperature, aligning with established patterns in insect biology (Atkinson, 1994). Intriguingly, we identified an interaction between temperature and development time, highlighting nuanced responses in body size. Despite limited differential gene expression in response to thermal tests, we observed evidence of a cold acclimation effect on cold tolerance. The mechanisms on a gene level in both tolerance and acclimation appear to be broad and complex, requiring further investigation. Gene expression analysis revealed temperature-dependent variations in larvae and pupae,

indicating potentially distinct mechanisms at play in response to temperature, but the lack of specificity in enriched GO terms, suggests broad changes in gene expression regulation. In adults, heat-tolerant individuals were observed regardless of rearing temperature and exhibited differential gene expression with enriched GO terms in response to heat centred around heat shock proteins, which could suggest that perhaps heat adaptation is constrained to particular pathways and or has particular long-term consequences (Feder *et al.*, 1992; Krebs and Loeschcke, 1994). This may explain why physiological mechanisms underpinning heat tolerance are centred around heat shock proteins (González-Tokman *et al.*, 2020). Our findings contribute to understanding the intricate interplay between temperature, development, and gene expression in tropical butterfly populations, shedding light on the adaptive strategies employed in the face of changing thermal conditions.

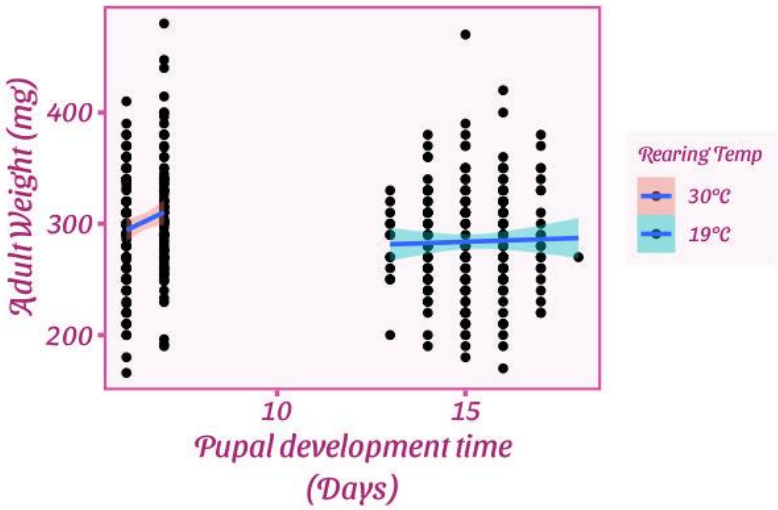
Acknowledgements

Thank you to Dr Yacine Ben Chehida for lending his expertise with Linux and bioinformatics and Dr Victoria J Lloyd for providing the starter scripts for the initial alignment, quantification and later BLASTx. I also thank Luke Richardson for his help with cold tolerance testing, and Xiyu Liu for her help in rearing an additional batch of samples.

Supplementary information

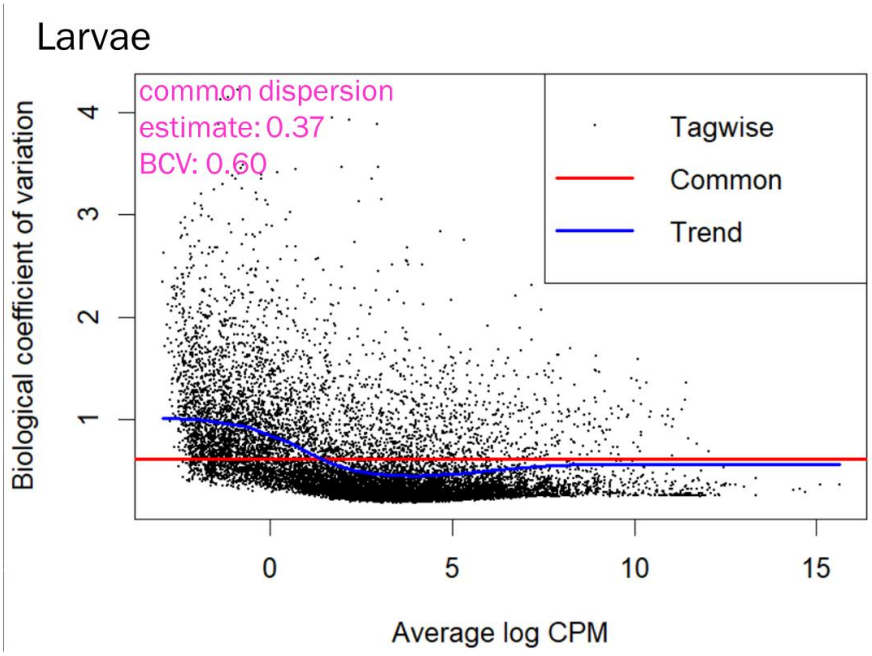


Supplementary figure 1: The trend of pupa weight by larval developmental time when reared at 30°C (red) and 19°C (blue).

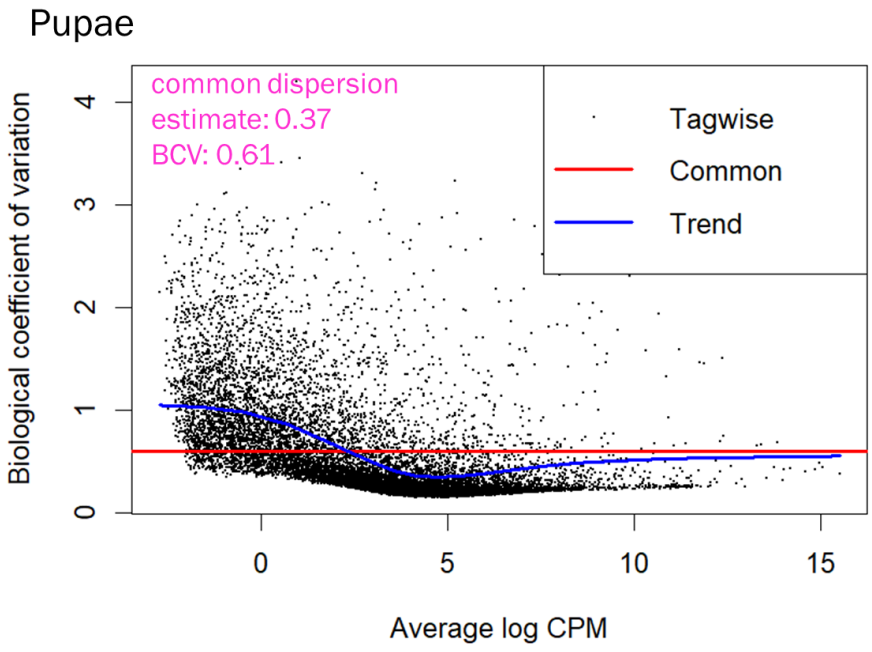


Supplementary Figure 2: The trend of adult weight by pupal developmental time when reared at 30°C (red) and 19°C (blue).

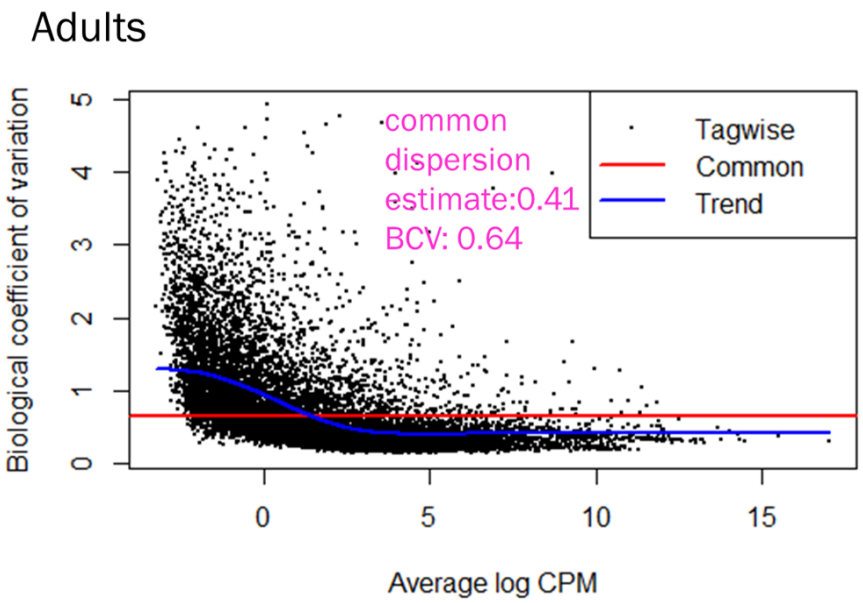
Biological coefficient of variation graphs (BCV)



Supplementary figure 2: A graph showing the Biological coefficient of variation (BCV) with also values of common dispersion in larvae samples



Supplementary figure 3: A graph showing the Biological coefficient of variation (BCV) with also values of common dispersion in pupae samples



Supplementary figure 4: A graph showing the Biological coefficient of variation (BCV) with also values of common dispersion in adult samples

GSEA all significant GO terms

Larvae

30°C vs 19°C – down regulated genes	
Category	**Term**
GOTERM_CC_DIRECT	GO:0000786~nucleosome
GOTERM_BP_DIRECT	GO:0006334~nucleosome assembly
GOTERM_BP_DIRECT	GO:0006325~chromatin organization
GOTERM_CC_DIRECT	GO:0005615~extracellular space
GOTERM_BP_DIRECT	GO:0005975~carbohydrate metabolic process
GOTERM_CC_DIRECT	GO:0005576~extracellular region
GOTERM_CC_DIRECT	GO:0005793~endoplasmic reticulum-Golgi intermediate compartment
GOTERM_CC_DIRECT	GO:0005789~endoplasmic reticulum membrane
GOTERM_BP_DIRECT	GO:0006886~intracellular protein transport
GOTERM_MF_DIRECT	GO:0008010~structural constituent of chitin-based larval cuticle
GOTERM_CC_DIRECT	GO:0016021~integral component of membrane
GOTERM_BP_DIRECT	GO:0008363~larval chitin-based cuticle development
GOTERM_MF_DIRECT	GO:0046982~protein heterodimerization activity
GOTERM_BP_DIRECT	GO:0032504~multicellular organism reproduction
GOTERM_MF_DIRECT	GO:0022857~transmembrane transporter activity
GOTERM_BP_DIRECT	GO:0006888~ER to Golgi vesicle-mediated transport

GOTERM_BP_DIRECT	GO:0055085~transmembrane transport
GOTERM_CC_DIRECT	GO:0016020~membrane
GOTERM_BP_DIRECT	GO:0008643~carbohydrate transport
GOTERM_CC_DIRECT	GO:0030134~ER to Golgi transport vesicle
GOTERM_CC_DIRECT	GO:0005795~Golgi stack
GOTERM_MF_DIRECT	GO:0008061~chitin binding
GOTERM_MF_DIRECT	GO:0042302~structural constituent of cuticle
GOTERM_CC_DIRECT	GO:0000139~Golgi membrane
GOTERM_CC_DIRECT	GO:0005777~peroxisome
GOTERM_CC_DIRECT	GO:0008250~oligosaccharyltransferase complex
GOTERM_CC_DIRECT	GO:0005794~Golgi apparatus

30°C vs 19°C – up regulated genes	
Category	**Term**
GOTERM_BP_DIRECT	GO:0045910~negative regulation of DNA recombination
GOTERM_BP_DIRECT	GO:0016584~nucleosome positioning
GOTERM_CC_DIRECT	GO:0022625~cytosolic large ribosomal subunit
GOTERM_BP_DIRECT	GO:0051276~chromosome organization
GOTERM_MF_DIRECT	GO:0031490~chromatin DNA binding
GOTERM_BP_DIRECT	GO:0006412~translation
GOTERM_CC_DIRECT	GO:0022627~cytosolic small ribosomal subunit
GOTERM_MF_DIRECT	GO:0031492~nucleosomal DNA binding
GOTERM_BP_DIRECT	GO:0030261~chromosome condensation
GOTERM_BP_DIRECT	GO:0031507~heterochromatin assembly
GOTERM_BP_DIRECT	GO:0007271~synaptic transmission, cholinergic
GOTERM_CC_DIRECT	GO:0042734~presynaptic membrane
GOTERM_CC_DIRECT	GO:0000786~nucleosome
GOTERM_CC_DIRECT	GO:0045211~postsynaptic membrane
GOTERM_MF_DIRECT	GO:0003723~RNA binding
GOTERM_MF_DIRECT	GO:0030527~structural constituent of chromatin
GOTERM_CC_DIRECT	GO:0000785~chromatin
GOTERM_BP_DIRECT	GO:0006334~nucleosome assembly
GOTERM_MF_DIRECT	GO:0003676~nucleic acid binding
GOTERM_MF_DIRECT	GO:0022848~acetylcholine-gated cation-selective channel activity
GOTERM_MF_DIRECT	GO:0071855~neuropeptide receptor binding

24°C vs 19°C – down regulated genes	
Category	**Term**
GOTERM_CC_DIRECT	GO:0033181~plasma membrane proton-transporting V-type ATPase complex
GOTERM_CC_DIRECT	GO:0005793~endoplasmic reticulum-Golgi intermediate compartment
GOTERM_CC_DIRECT	GO:0005789~endoplasmic reticulum membrane
GOTERM_CC_DIRECT	GO:0016021~integral component of membrane
GOTERM_CC_DIRECT	GO:0030134~ER to Golgi transport vesicle
GOTERM_CC_DIRECT	GO:0016020~membrane
GOTERM_CC_DIRECT	GO:0016471~vacuolar proton-transporting V-type ATPase complex
GOTERM_CC_DIRECT	GO:0005777~peroxisome
GOTERM_CC_DIRECT	GO:0030126~COPI vesicle coat
GOTERM_BP_DIRECT	GO:0006890~retrograde vesicle-mediated transport, Golgi to ER
GOTERM_BP_DIRECT	GO:0006891~intra-Golgi vesicle-mediated transport
GOTERM_CC_DIRECT	GO:0000139~Golgi membrane
GOTERM_CC_DIRECT	GO:0033179~proton-transporting V-type ATPase, V0 domain
GOTERM_CC_DIRECT	GO:0000220~vacuolar proton-transporting V-type ATPase, V0 domain
GOTERM_CC_DIRECT	GO:0005788~endoplasmic reticulum lumen
GOTERM_MF_DIRECT	GO:0038024~cargo receptor activity
GOTERM_BP_DIRECT	GO:0007035~vacuolar acidification
GOTERM_CC_DIRECT	GO:0000221~vacuolar proton-transporting V-type ATPase, V1 domain
GOTERM_BP_DIRECT	GO:0061355~Wnt protein secretion
GOTERM_BP_DIRECT	GO:0006886~intracellular protein transport
GOTERM_CC_DIRECT	GO:0008250~oligosaccharyltransferase complex
GOTERM_BP_DIRECT	GO:0010883~regulation of lipid storage
GOTERM_CC_DIRECT	GO:0005794~Golgi apparatus
GOTERM_BP_DIRECT	GO:0055085~transmembrane transport
GOTERM_MF_DIRECT	GO:0022857~transmembrane transporter activity
GOTERM_BP_DIRECT	GO:0006487~protein N-linked glycosylation
GOTERM_CC_DIRECT	GO:0005615~extracellular space
GOTERM_BP_DIRECT	GO:0015031~protein transport
GOTERM_MF_DIRECT	GO:0015078~hydrogen ion transmembrane transporter activity
GOTERM_BP_DIRECT	GO:0018279~protein N-linked glycosylation via asparagine
GOTERM_CC_DIRECT	GO:0005791~rough endoplasmic reticulum

30°C vs 24°C – up-regulated genes

Category	Term
GOTERM_CC_DIRECT	GO:0000220~vacuolar proton-transporting V-type ATPase, V0 domain
GOTERM_CC_DIRECT	GO:0000221~vacuolar proton-transporting V-type ATPase, V1 domain
GOTERM_CC_DIRECT	GO:0016021~integral component of membrane
GOTERM_CC_DIRECT	GO:0048471~perinuclear region of cytoplasm

30°C vs 24°C – down-regulated genes	
Category	**Term**
GOTERM_MF_DIRECT	GO:0042302~structural constituent of cuticle
GOTERM_CC_DIRECT	GO:0031012~extracellular matrix
GOTERM_BP_DIRECT	GO:0032504~multicellular organism reproduction
GOTERM_MF_DIRECT	GO:0004252~serine-type endopeptidase activity
GOTERM_BP_DIRECT	GO:0006508~proteolysis
GOTERM_BP_DIRECT	GO:0005975~carbohydrate metabolic process
GOTERM_BP_DIRECT	GO:0008365~adult chitin-based cuticle development
GOTERM_MF_DIRECT	GO:0004568~chitinase activity
GOTERM_MF_DIRECT	GO:0004497~monooxygenase activity

Pupae

30°C vs 19°C – down-regulated genes	
Category	**Term**
GOTERM_MF_DIRECT	GO:1902936~phosphatidylinositol bisphosphate binding
GOTERM_BP_DIRECT	GO:0032504~multicellular organism reproduction
GOTERM_MF_DIRECT	GO:0008061~chitin binding
GOTERM_CC_DIRECT	GO:0005576~extracellular region

30°C vs 19°C – up-regulated genes	
Category	**Term**
GOTERM_BP_DIRECT	GO:0045910~negative regulation of DNA recombination
GOTERM_BP_DIRECT	GO:0051276~chromosome organization
GOTERM_MF_DIRECT	GO:0031492~nucleosomal DNA binding
GOTERM_BP_DIRECT	GO:0030261~chromosome condensation
GOTERM_BP_DIRECT	GO:0031507~heterochromatin assembly
GOTERM_MF_DIRECT	GO:0030527~structural constituent of chromatin
GOTERM_BP_DIRECT	GO:0006334~nucleosome assembly

GOTERM_MF_DIRECT	GO:0003677~DNA binding
GOTERM_BP_DIRECT	GO:0042026~protein refolding
GOTERM_CC_DIRECT	GO:0005634~nucleus

Adult butterflies

30°C vs 19°C – down-regulated genes	
Category	**Term**
GOTERM_CC_DIRECT	GO:0022626~cytosolic ribosome
GOTERM_CC_DIRECT	GO:0005747~mitochondrial respiratory chain complex I
GOTERM_CC_DIRECT	GO:0022625~cytosolic large ribosomal subunit
GOTERM_CC_DIRECT	GO:0005743~mitochondrial inner membrane
GOTERM_BP_DIRECT	GO:0032981~mitochondrial respiratory chain complex I assembly
GOTERM_CC_DIRECT	GO:0022627~cytosolic small ribosomal subunit
GOTERM_BP_DIRECT	GO:1902600~hydrogen ion transmembrane transport
GOTERM_BP_DIRECT	GO:0032543~mitochondrial translation
GOTERM_BP_DIRECT	GO:0040003~chitin-based cuticle development
GOTERM_CC_DIRECT	GO:0005762~mitochondrial large ribosomal subunit
GOTERM_MF_DIRECT	GO:0046961~proton-transporting ATPase activity, rotational mechanism
GOTERM_BP_DIRECT	GO:0006120~mitochondrial electron transport, NADH to ubiquinone
GOTERM_MF_DIRECT	GO:0015078~hydrogen ion transmembrane transporter activity
GOTERM_BP_DIRECT	GO:0006099~tricarboxylic acid cycle
GOTERM_CC_DIRECT	GO:0033181~plasma membrane proton-transporting V-type ATPase complex
GOTERM_MF_DIRECT	GO:0008010~structural constituent of chitin-based larval cuticle
GOTERM_MF_DIRECT	GO:0046933~proton-transporting ATP synthase activity, rotational mechanism
GOTERM_CC_DIRECT	GO:0005759~mitochondrial matrix
GOTERM_MF_DIRECT	GO:0008137~NADH dehydrogenase (ubiquinone) activity
GOTERM_BP_DIRECT	GO:0015986~ATP synthesis coupled proton transport
GOTERM_BP_DIRECT	GO:0006313~transposition, DNA-mediated
GOTERM_BP_DIRECT	GO:0007005~mitochondrion organization
GOTERM_CC_DIRECT	GO:0015935~small ribosomal subunit
GOTERM_MF_DIRECT	GO:0051539~4 iron, 4 sulfur cluster binding
GOTERM_MF_DIRECT	GO:0009055~electron carrier activity
GOTERM_CC_DIRECT	GO:0005751~mitochondrial respiratory chain complex IV

GOTERM_CC_DIRECT	GO:0000276~mitochondrial proton-transporting ATP synthase complex, coupling factor F(o)
GOTERM_CC_DIRECT	GO:0000221~vacuolar proton-transporting V-type ATPase, V1 domain
GOTERM_CC_DIRECT	GO:0005763~mitochondrial small ribosomal subunit

30°C vs 19°C – up-regulated genes	
Category	**Term**
GOTERM_MF_DIRECT	GO:0003723~RNA binding
GOTERM_CC_DIRECT	GO:0032040~small-subunit processome
GOTERM_BP_DIRECT	GO:0016584~nucleosome positioning
GOTERM_BP_DIRECT	GO:0045910~negative regulation of DNA recombination
GOTERM_CC_DIRECT	GO:0005829~cytosol
GOTERM_MF_DIRECT	GO:0003724~RNA helicase activity
GOTERM_BP_DIRECT	GO:0051276~chromosome organization
GOTERM_BP_DIRECT	GO:0008033~tRNA processing
GOTERM_MF_DIRECT	GO:0003690~double-stranded DNA binding
GOTERM_CC_DIRECT	GO:0071011~precatalytic spliceosome
GOTERM_BP_DIRECT	GO:0000398~mRNA splicing, via spliceosome
GOTERM_CC_DIRECT	GO:0071013~catalytic step 2 spliceosome
GOTERM_BP_DIRECT	GO:0032259~methylation
GOTERM_CC_DIRECT	GO:0005634~nucleus

Rearing temperature 19°C **Heat no KO vs Control up-regulated genes**	
Category	**Term**
GOTERM_MF_DIRECT	GO:0031072~heat shock protein binding
GOTERM_BP_DIRECT	GO:0034605~cellular response to heat
GOTERM_BP_DIRECT	GO:0061077~chaperone-mediated protein folding
GOTERM_MF_DIRECT	GO:0051787~misfolded protein binding
GOTERM_BP_DIRECT	GO:0009408~response to heat
GOTERM_MF_DIRECT	GO:0016887~ATPase activity
GOTERM_BP_DIRECT	GO:0051085~chaperone mediated protein folding requiring cofactor
GOTERM_MF_DIRECT	GO:0051879~Hsp90 protein binding

Rearing temperature 19°C **Heat no KO vs KO up-regulated genes**

Category	Term
GOTERM_BP_DIRECT	GO:0034605~cellular response to heat
GOTERM_BP_DIRECT	GO:0061077~chaperone-mediated protein folding
GOTERM_MF_DIRECT	GO:0051087~chaperone binding
GOTERM_BP_DIRECT	GO:0009408~response to heat
GOTERM_BP_DIRECT	GO:0051085~chaperone mediated protein folding requiring cofactor

Rearing temperature 30°C Heat no KO vs Control up-regulated genes	
Category	**Term**
GOTERM_MF_DIRECT	GO:0051087~chaperone binding
GOTERM_BP_DIRECT	GO:0061077~chaperone-mediated protein folding
GOTERM_BP_DIRECT	GO:0051085~chaperone mediated protein folding requiring cofactor
GOTERM_BP_DIRECT	GO:0034620~cellular response to unfolded protein
GOTERM_BP_DIRECT	GO:0034605~cellular response to heat
GOTERM_MF_DIRECT	GO:0051787~misfolded protein binding
GOTERM_BP_DIRECT	GO:0009408~response to heat
GOTERM_MF_DIRECT	GO:0044183~protein binding involved in protein folding
GOTERM_MF_DIRECT	GO:0051879~Hsp90 protein binding
GOTERM_MF_DIRECT	GO:0016887~ATPase activity

Rearing temperature 30°C Heat no KO vs KO up-regulated genes	
Category	**Term**
GOTERM_MF_DIRECT	GO:0031072~heat shock protein binding
GOTERM_BP_DIRECT	GO:0061077~chaperone-mediated protein folding
GOTERM_BP_DIRECT	GO:0051085~chaperone mediated protein folding requiring cofactor
GOTERM_MF_DIRECT	GO:0051879~Hsp90 protein binding
GOTERM_BP_DIRECT	GO:0034620~cellular response to unfolded protein
GOTERM_BP_DIRECT	GO:0034605~cellular response to heat
GOTERM_MF_DIRECT	GO:0051787~misfolded protein binding
GOTERM_MF_DIRECT	GO:0044183~protein binding involved in protein folding

Chapter 3

Disentangling local adaptation and phenotypic plasticity in traits associated with altitude and thermal adaptation in widespread tropical butterflies

Tien T. T. Nguyen[1]†, Patricio A. Salazar[2]†, Sophie H. Smith[3], Kimberly G. Gavilanes[4], Michelle Guachamín[4], Gabriela Montejo-Kovacevich[5], Luke B. Richardson1[6], Caroline N. Bacquet[4], Nicola J. Nadeau[1]*

†Authors with equal contribution

*Corresponding author

Email: n.nadeau@sheffield.ac.uk

[1]School of Biosciences, University of Sheffield, Alfred Denny Building, Western bank, Sheffield S10 2TN, UK

[2]Wellcome Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Saffron Walden CB10 1RQ

[3]Centre for Molecular Biodiversity Research, Leibniz Institute for the Analysis of Biodiversity Change, Museum Koenig Bonn, Adenauerallee 127, 53113 Bonn, Germany

[4]Universidad Regional Amazónica IKIAM, Kilómetro 7 Vía Muyuna, Ciudad de Tena, 150102, Tena, Napo, Ecuador

[5]Department of Zoology, University of Cambridge, Cambridge, CB2 3EJ, United Kingdom

[6]Department of Geography, University of Sheffield, Winter St, Sheffield S3 7ND

Author contributions

This chapter was written in manuscript format for future submission.

Data collection with regard to capture, rearing the thermal tolerance testing was conducted by Patricio A. Salazar, Sophie H. Smith, Kimberly G. Gavilanes, Michelle Guachamín and Nicola J. Nadeau. Photographing, data collection and partial analysis regarding wing traits, were assisted by Gabriela Montejo-Kovacevich and Luke B. Richardson. Tien T. T. Nguyen wrote the manuscript and analysed the data. Patricio A. Salazar conducted the in data analysis of RTE developmental traits, preparation of the data analyses section of the methods and partial preparation of the introduction and other methods. Sophie H. Smith prepared the full thermal tolerance methodology in SI. Nicola J. Nadeau assisted in the partial preparation of the discussion. Patricio A. Salazar and Nicola J. Nadeau contributed to the project design. I wrote the manuscript with feedback from Nicola Nadeau

Abstract

Climatic stratifications, in particular differences in temperature, occur along altitudinal clines. This can lead to local adaptation and genetic divergence between populations at different altitudes. Understanding genetic and phenotypic divergence across these regions can give insight into speciation and diversification, as well as aid in our knowledge of how species may respond to possible climate change scenarios. The majority of past research has focused on temperate regions, yet it is the tropics that are thought to be the most vulnerable to rising temperatures. In addition, year-round stable temperatures in the tropics make altitudinal temperature variation more pronounced and increase the likelihood of local adaptation across relatively narrow gradients. Here we focus on two mimetic butterfly species, *Heliconius erato* and *Heliconius melpomene* which are widespread across the neotropics and occur along the altitudinal slope of the Andes. We demonstrate through 'common garden' rearing of offspring from wild females caught along an altitudinal gradient, as well as rearing of high and low altitude populations in their reciprocal temperature environments, evidence of altitudinal selection as well gene-by-environment reactions with temperature in: developmental, morphological, and thermal tolerance traits, with support for significant heritabilities of these traits. The traits that exhibited local adaptation were distinct in either species, illustrating how identical selection factors, within the same environments, can variably influence population dynamics and contribute to the process of speciation. *H. erato*, in particular, showed strong evidence of adaptive plasticity in response to heat, with increased heat knockout times at higher rearing temperatures. This has positive implications for this species under warming conditions, but the same was not observed for *H. melpomene*.

Introduction

Ever since Humboldt (1807) (Romanowski and Jackson, 2009) drew the attention of the scientific world to the recurrent morphologies that plants show along altitudinal gradients, no matter where in the World these occur, we have considered altitude as a fundamental factor driving the diversity of form and function observed in terrestrial organisms. With consistent stratification in climatic conditions, altitude can lead to local adaptation and genetic divergence between populations at different altitudes, which in turn can result in speciation and diversification. However, despite its importance to our understanding of the origins of biological diversity across the globe, altitudinal adaptation has mostly been studied in temperate organisms (Hodkinson, 2005), even though its impact on speciation and diversification is likely to be more important in the tropics (Janzen, 1967). In the tropics, climate not only varies rapidly with altitude but—due to the lack of seasonality—it varies little throughout the year, creating—as a result—relatively narrow and stable climatic strata (Janzen, 1967), which are likely responsible for the huge diversity and turnover in species composition along tropical altitudinal gradients. Despite its importance to our understanding of the evolutionary processes that lead to altitudinal adaptation, and the origins and maintenance of tropical diversity, the causes and consequences of altitudinal adaptation in tropical organisms have still attracted relatively little attention (Slade and Ong, 2023).

Altitude, the vertical distance from sea-level to the top of the highest mountains, causes major and consistent changes in three key climatic features: the average ambient temperature, atmospheric pressure, and the intensity of solar radiation (Körner, 2007). These changes, in turn, cause living organisms to adapt to these changing conditions.

For example, many lepidopteran species have shown evidence of increased body size with altitude (Sullivan and Miller, 2007) due to the temperature decrease at higher altitudes in accordance with Bergmann's rule (1848). Whether this is an adaptive advantage or just a general plastic consequence of increased developmental time has been up for debate, with perhaps stronger arguments for the latter (Teplitsky and Millien, 2014). There is a lack of evidence to determine whether being smaller in warmer environments is advantageous as larger body sizes have shown to aid in buffering against heat stress as seen in *Bicyclus anynana* (Klockmann, Günter and Fischer, 2017). However, there is evidence of an adaptive advantage of being larger in colder environments where larger body sizes appear to increase cold tolerance (Scharf, Sbilordo and Martin, 2014).

Butterfly wing morphologies have also shown variation with altitude such as: aspect ratio (Montejo-Kovacevich *et al.*, 2019; Montejo-Kovacevich *et al.*, 2021) and wing loading (Nève and Després, 2020) in association with flight performance with atmospheric pressure; and wing colouration associated with solar intensity and thermoregulation in compliance with the thermal melanism hypothesis (TMH) (Clusella Trullas, van Wyk and Spotila, 2007). For example, *Catasticta* butterfly species have been shown to darken at higher altitudes, which was linked to thermoregulation (and therefore the TMH) as it increased their rates of heating up (Dufour *et al.*, 2018).

Depending on the trait of interest and its ecological role, the observed altitudinal variation in a phenotypic trait can be the outcome of three modes of adaptation. These include: local (genetic) adaptation through natural selection; plasticity in developmental, physiological, morphological or behavioural responses to the environmental conditions; or a combination of both via a genotype-by-environment interaction (GXE), where the

extent or direction of genetically controlled plasticity differs between populations (Fox *et al.*, 2019).

Evidence of local adaptation that leads to genetic and heritable differences between populations at different altitudes has been observed in many butterfly species, such as in temperate butterfly *Lycaena tityrus,* which when reared in 'common garden' (standardised) conditions, presents a range of traits that vary between populations. Highland compared to lowland populations were shown to have longer development time, increased cold but decreased heat-stress resistance, and increased flight duration (Karl *et al.*, 2008). While in montane butterfly *Colias eriphyle* wing solar absorptivity exhibits variation with altitude, but this is due to plasticity, dependent upon developmental temperature (Kingsolver and Buckley, 2017). Natural variation in trait values can often be an interplay between some degree of adaptive genetic divergence between populations coupled with some degree of adaptive phenotypic plasticity. This been observed in butterflies such as *Melitaea cinxia* with regard to metabolic rates (Niitepõld, 2010) and in *Bicyclus anynana* with developmental growth rates (Brakefield and Kesbeke, 1997) where in both species, the trait present is dependent upon population in interaction with rearing temperature.

A full understanding of the nature of the phenotypic variation observed in the wild is indispensable, not only to understand the evolutionary processes of adaptation, speciation and diversification, but also learn how living organisms may cope with long-term climatic changes (such as global warming) that are substantially altering the climate around the globe.

Here we studied the relative roles of genetics and plasticity in determining morphological, developmental, and thermal tolerance variation across an altitudinal gradient, in two well-studied species of *Heliconius* butterflies (*H. erato* and *H. melpomene*) on the Eastern slopes of the Andes in Ecuador (Figure 1).

The co-mimicking species *H. erato* and *H. melpomene* have a wide distribution across the neotropics and occupy a range of temperatures. They have been extensively studied and are famously known for their variable colour patterning and müllerian mimicry (Jiggins, 2017). Therefore, the tools and resources available make them an excellent model system for studying altitudinal adaptation in the tropics. However, differences between populations of *Heliconius* in thermal adaptation have been relatively understudied. Initial evidence of altitudinal adaptations within these species have thus far revealed differences in wing aspect ratio, which appears to have a genetic basis (Montejo-Kovacevich *et al.*, 2019; Montejo-Kovacevich *et al.*, 2021, 2022) and some variation in heat tolerance, which may be mainly due to plasticity (Montejo-Kovacevich *et al.*, 2020). Therefore, through controlled temperature rearing experiments we aimed to disentangle the roles of altitude and temperature (plasticity) in these species with regard to their developmental traits, and thermal tolerance.

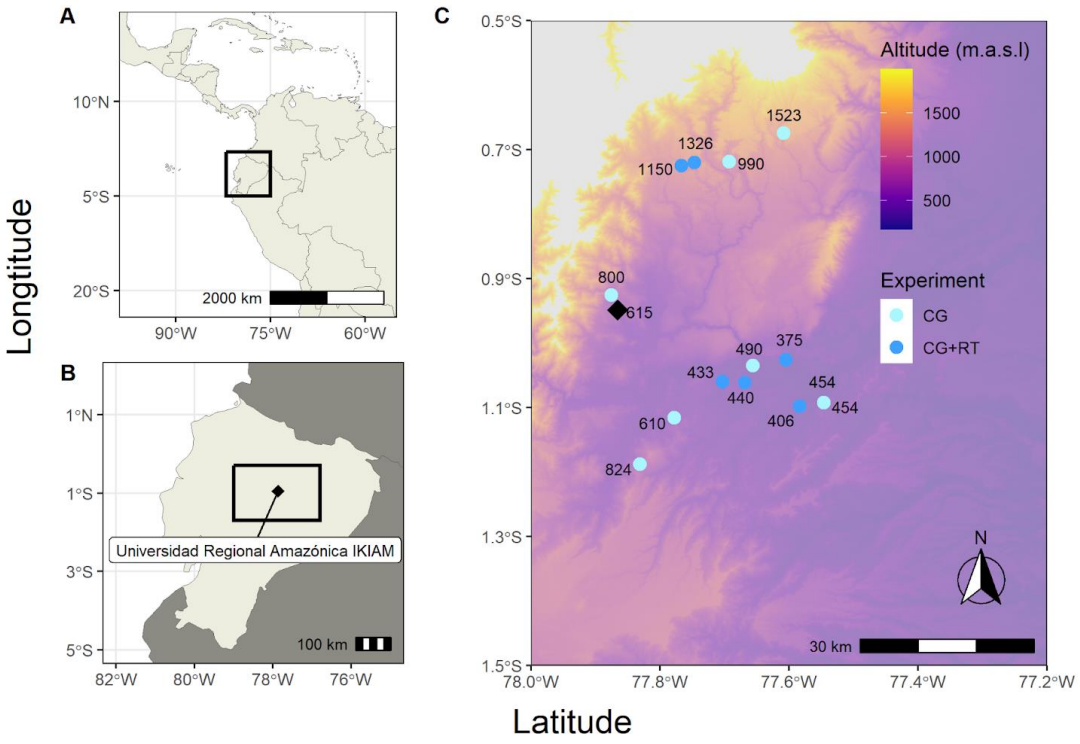


Figure 1: Capture sites and rearing location of butterflies sampled for the 'Common Garden' (CG) and 'Reciprocal Transplant' (RT) experiments. Location of the study within South America (A) and Ecuador (B) showing the Universidad Regional Amazonica IKIAM, where experiments were conducted, C) Locations of the rearing site (IKIAM, black diamond) and capture sites (dark and pale blue points, representing sampling sites used for both experiments and the common garden experiment only, respectively) with altitude of each point given next to it and altitude of the region shown by background colours. Sampling site altitudes ranged from 375 metres above sea level (m.a.s.l) to 1523 m.a.s.l.

Materials and Methods

Experimental design

Two separate experiments were conducted to investigate the relative roles of local (genetic) adaptation and phenotypic plasticity in the two studied species: *Heliconius erato*

and *H. melpomene*. The first experiment, a Common Garden Experiment (CGE), involved rearing under a common environment the offspring of wild-caught females from both species, collected across a continuous altitude gradient ranging from 380m to 1250m above sea level for *H. erato* and 380m to 1500m for *H. melpomene* (Figure 1C). Rearing was carried out under controlled laboratory conditions at the Amazonian Regional University Ikiam (600m above sea level) (Figure 1B). The lab temperature was set to approximately intermediate values between the extreme temperatures experienced across the sampled altitudinal range: 21.2 ± 1.1 °C (mean ± sd). Humidity was monitored but not strictly controlled, and the light regime mimicked natural day-night cycles (~12:12 hours).

The second experiment, a Reciprocal Transplant Experiment (RTE), involved rearing offspring from wild-caught females from two altitude extremes, approximately 400 ± 50m and 1200 ± 100m above sea level (Figure 1C). In contrast to the CGE, the offspring from both altitudes were reared in growth chambers under two temperature treatments: 19°C and 24°C, representing the average conditions in the forest understory of highland and lowland altitudes respectively. This was, therefore, a fully-crossed design between rearing temperature and altitude. We were able to replicate the natural conditions well: 19.1 ± 1.7 °C at ~1200m and 23.5 ± 2.1 °C at ~400m, with a day/night fluctuation (Montejo-Kovacevich *et al.*, 2020). A 12:12 hours day-night regime was implemented in both growth chambers.

Collection sites for the mothers were limited to within the geographical range of the co-mimetic subspecies *H. erato lativitta* and *H. melpomene malleti*, to avoid any potential confounding effects of variation associated with colour pattern in other subspecies.

Caterpillars were provided with a cutting of a suitable food plant for each species (*Passiflora punctata* for *H. erato* and *P. edulis* for *H. melpomene*) and allowed to feed *ad libitum*. Traits associated with development, thermal tolerance, and wing morphology were measured for each reared individual.

Overall, by rearing individuals from populations across a range of altitudes in a common environment, the CGE allowed us to assess the heritable effects of altitude without the effects that varying conditions at different altitudes could have on trait development. On the other hand, by comparing traits of individuals from the same altitude but reared at different temperatures, and—conversely—comparing traits of individuals from distinct altitudes but reared in the same temperature, the RTE allowed us to tease apart the effects of rearing conditions (phenotypic plasticity) from heritable population differences (local genetic adaptation). In addition, due to its crossed design, the RTE allowed us to assess potential genotype-by-environment interactions (implying both phenotypic plasticity and heritable population differences on the same trait), when individuals from different altitudes showed distinct responses in the two alternative temperature treatments.

Developmental parameters

Eggs were collected daily in the late afternoon at the same time. Eggs, larvae, and pupae checked daily throughout the experiments to accurately record mortality, and developmental time from hatching to: moulting into 5th instar larvae, pupation and eclosion into adult butterflies. Pupa weight was measured one day after pupation, and adult weight was measured one day after eclosion before feeding.

Thermal tolerance

Adult offspring were tested for their tolerance to thermal extremes of hot and cold. All thermal tolerance tests were carried out in the 'common garden' rearing lab at 21°C, and researchers carrying out the experiment were blind to the butterfly's altitude of origin.

For the adults of the CGE, cold tolerance tests were conducted on the second day after eclosion from the pupa and heat tolerance tests were conducted the following day. Whereas for the RTE, butterflies were randomly assigned to either the cold or heat tolerance test (not both) conducted on the second day after eclosion (due to the RTE individuals being used for a subsequent gene expression study).

Cold tolerance was tested by exposing the butterflies to 5 ± 1 °C for 10 minutes, measuring the time taken for the butterfly to exhibit chill coma response (Semper, 1883) (or time to cold knockout (KO)) and then the time taken to recover, which was characterised by the ability to support itself on 3 or more legs.

Heat tolerance was tested as heat knockout (KO) time at 39°C (± 1°C) (and recovery, in common garden only) following the methods of (Montejo-Kovacevich *et al.*, 2020), with minor modification (See Supplementary information). After the heat tolerance experiment, all butterflies were killed and preserved. See Supplementary information for full details on thermal tolerance testing methodology

Wing Measurements

Detached wings were photographed dorsally with a Nikon D7000 APS-C DSLR with fixed settings of: 40 mm f/2.8 lens, f/10, 1/60, ISO 100, under standardised conditions with an

X-Rite passport colorchecker and ruler included. Damage to the wings was assessed by eye, with wings showing more than 10% damage removed from our analyses for the GCE samples, but all samples were kept in RTE as long as the whole wing segment was present. Custom scripts were developed in Adobe Photoshop 2021 to automate colour correction, and cropping of each wing segment.

Wing size, and shape were measured from preserved wings analysed as in the methods in (Montejo-Kovacevich *et al.*, 2019). 'Wing load' was measured by dividing total adult weight (mg) by wing area (mm^2) to give mg of weight per mm wing (mg/mm^2).

Black wing proportion as well as red brightness were obtained using the 'ColourDistance' R package through a K-means clustering approach (See Supplementary Figure 1). We specified that the images contained three distinct colours, as both species have distinct patches of black, red and yellow. ColourDistance clusters together all pixels that are the most similar to one another into the defined number of groups, while calculating the proportion and mean R.G.B values for each group (Weller and Westneat, 2019). Brightness of the red region (hereafter red brightness) was calculated by combining mean R.G.B values in the red region and dividing three to get an estimate of percentage reflectance (within the range of the camera sensitivity).

Data analyses

Variation in each measured trait was modelled using Linear Mixed-Effect or Generalised Linear Mixed-Effect Modelling depending on the statistical nature of the trait (e.g., Gaussian distribution for morphological measures like body mass and Gamma distribution for 'waiting-time' variables such as development times).

Explanatory variables incorporated in the models varied depending on the experiment and the trait analysed. In both experiments, the set of explanatory variables of interest was implemented explicitly in the experimental design. In the CGE, 'altitude' (modelled as a continuous variable) was the key effect of interest, while in the RTE 'rearing temperature' (19°C and 24°C treatments), 'altitude' (categorical: 400m and 1200m) and the statistical interaction between them were the effects of interest. Additional potentially explanatory variables were considered for each trait; for instance, 'sex' for traits measured in adults—which can be sexed—or 'observer identity' for traits affected by subjective observer bias (e.g., cold- and heat-knockdown times). In addition, because in both experiments a variable number of offspring of the same mother were reared, we modelled the effect of relatedness by including mother as a random effect. This random effect not only allowed us to account for the lack of statistical independence between offspring of the same mother (full sibs), but also allowed us to estimate heritabilities for the measured traits (see below).

For each trait, we followed the same modelling protocol. We first generated a list of nested candidate models combining all explanatory variables plus any pairwise statistical interaction considered relevant for each trait (for the RTE, the interaction between 'rearing temperature' and 'altitude' was always incorporated, since it was a key parameter of interest).

We then fit the candidate models to the data, ranked them according to their AICc scores (Burnham and Anderson, 2004), and selected the model with the lowest AICc value that would also contain the set of explanatory variables of interest in each experiment. We used this selected model to estimate the effect sizes of interest in each experiment. The

model selection process was also used to assess which potentially explanatory variables showed evidence of an effect, and which did not. Explanatory variables with a likely effect on a trait were taken as those contained in the model with the lowest AICc (which we term the "top ranked model", but not necessarily the one we selected to estimate effect sizes), or in the set of models ranked within two AICc units from the top ranked (Burnham and Anderson, 2004). Model fitting was carried out using the *R* package *lme4* (v.1.1-31;(Bates *et al.*, 2015)), and model selection facilitated with *AICcmodavg* (v.2.3-2, (Mazerolle, 2023)).

With the CGE data only, the random effect of 'mother' estimated with the selected model was used to calculate the intraclass correlation coefficient (ICC) between offspring of the same mother (following Nakagawa and Schielzeth, (2010), using package *rptR* (v.0.9.22 (Stoffel, Nakagawa and Schielzeth, 2017)). This ICC was in turn used to estimate each trait's heritability as 2 * ICC , measuring the upper limit of heritability, based on Falconer and Mackay (1996). Re-mating is rare in the wild in the two species studied (Walters *et al.*, 2012), so we assume that individuals from the same mother are full siblings.

Finally, to compare the effects of altitude on the various traits measured in the CGE, we recalculated the slopes of each trait in relation to altitude, after standardising the original trait values (mean = 0, sd = 1) and re-fitting the selected models. For visual comparisons, we plotted these standardised slopes (effect sizes in sd units) next to each other. For the RTE, in contrast, to compare the effects of rearing individuals from the same altitude in different temperatures (phenotypic plasticity), or to compare individuals from different altitudes reared in the same temperature (local genetic adaptation), we calculated the relevant effect sizes as the ratio between the estimated marginal means (as predicted by

the selected models) for individuals in the corresponding temperature-by-altitude combinations. These effect sizes were estimated using the package *emmeans* (Searle, Speed and Milliken, 2023a).

All statistical analyses were conducted in R version 4.+ (R core Team). All graphs were produced with 'ggplot2' (Wickham, 2016), and maps required additional packages "rnaturalearth" (Massicotte and South, 2023), "raster" (Hijmans, 2023), "sf" (Pebesma, 2018; Pebesma and Bivand, 2023), and "ggrepel" (Slowikowski, 2023).

Results

In the Common Garden Experiment (GCE) 29–45 families were analysed in *H. erato* with >400 individuals for development time and body size traits, 1549 for larval survival, 802 for pupal survival, and >300 for other traits across altitudes. The GCE in *H. melpomene* included 20–25 families, with >400 individuals per development time and body size traits, >800 for survival, and >300 for other traits across altitudes. In the reciprocal transplant experiment (RTE), >14 families were analysed in *H. erato* with >200 individuals for survival, and >100 for all other traits between both altitudes. The RTE for *H. melpomene* >6 families were analysed with >100 individuals for survival, >30 individuals for thermal tolerance and >50 individuals for all other traits.

It should be considered that in the RTE, *H. melpomene* had a much smaller sample size than *H. erato* especially in lowland broods causing a lack of power to discern a strong population or interaction with temperature effect in some traits. See supplementary tables 1 and 2 for full details of models, and the sample sizes included for each trait and experiment

There were a number of individuals that did not reach heat coma in during the maximum testing period; these are excluded from the main text analysis, as they had no 'true' time

to heat coma. However, additional analysis giving these non-knockout individuals an upper limit value of 128 minutes to heat coma time can be found in supplementary information.

Developmental Traits

Developmental time

In the GCE, altitude had no substantial effect on larval or pupal development time, top ranked models were the null models in both *H. erato* (larval AICcWt = 0.66, pupal AICcWt = 0.7) and *H. melpomene* (larval AICcWt = 0.68, pupal AICcWt = 0.7) (Figure 2: A and B). However, in the RTE, lowland populations of *H. erato* tended to develop faster as larvae regardless of rearing temperature (400m/1200m, effect size: 0.9x ± 0.09). The altitudinal effect was marginal, as the top ranked model contained both temperature and altitude (without an interaction) (AICcWt = 0.53, ΔAICc = 1.44) was not 2 AICc units smaller than the model with temperature alone (AICcWt = 0.26). The opposite trend was observed in the pupae, but only when reared at 19°C, where lowland populations took 1.06x ± 0.05 as long as than their highland counterparts, but there was no difference between populations when reared at 24°C (400m/1200m, effect size: 0.99x ± 0.05). However, this interaction between temperature and altitude was marginal as the model with this interaction term, while the highest ranked (AICcWt = 0.35), had been within 2 AICc units as the model with just temperature alone (AICcWt = 0.15, ΔAICc = 1.77). Overall, in *H. erato* there appeared to be no strong effect of altitude alone or in an interaction with temperature on development time. However, the effect of temperature alone was substantial, as larval development time halved at 24°C (24°C:19°C, effect size = 0.60x ± 0.03) as did pupal development time (24°C:19°C, effect size = 0.51x ± 0.02) in both populations (Figure 2C). This was similarly seen in *H. melpomene* with pupal

developmental time being halved at 24°C, (24°C:19°C, effect size = 0.51x ± 0.03) in both altitudes and with the model with temperature alone being the highest ranked (AICcWt = 0.46) (Figure 2D). However, in *H. melpomene* the highest ranked model for larval development time supported an interaction between altitude and temperature (AICcWt =0.78). While increased temperatures overall lead to faster development, this effect was stronger in lowland populations (24°C:19°C, effect size = 0.59x ± 0.04) than highland populations (24°C:19°C, effect size = 0.69x ± 0.05) (Figure 2D).

In regard to heritability of larval and pupal development times, they were significantly heritable in both *H. erato* (larval ICC = 0.37, p-value = 1.10E-44; pupal ICC =0.35, p-value =1.13E-31 (Figure 3A)) and *H. melpomene* (larval ICC = 0.22, p-value = 9.01E-12; pupal ICC =0.38, p-value =2.84E-23 (Figure 3B)).

Body Size

There was no difference in body size between butterflies from different altitudes (in both pupa and adult butterfly weights), when reared in the CGE (Figure 2A)). Highest ranked models only contained development time, in which longer development times resulted in larger body sizes in both *H. erato* (pupa AICcWt = 0.47, Adult AICcWt = 0.56), and *H. melpomene* (Pupa AICcWt = 0.68, Adult AICcWt = 0.3). In the RTE, there appeared to be a possible effect of altitude on body size in interaction with rearing temperature in both *H. erato* and *H. melpomene* (adult weight only) but this was not supported by the modelling. Highland *H. erato* pupae tended to be smaller at higher rearing temperatures (24°C:19°C, effect size = 0.83x ± 0.13), which was not seen in lowland pupae (24°C:19°C, effect size = 0.9x ± 0.13) (Figure 2C)). Whereas, *H. erato* adults, lowland individuals were smaller with higher rearing temperature (24°C:19°C, effect size = 0.82x ± 0.08) and there was no effect in highland adults (24°C:19°C, effect size = 0.87x ± 0.11) (Figure 2C)). Yet, the highest

ranked models for *H. erato* pupal weight, contained only temperature interacting with development time as explanatory factors (AICcWt = 0.46) and for adult weight, the highest ranked model had contained temperature, and development time with an interaction (AICcWt = 0.25) but, this model was not 2 AICc units lower model containing temperature alone (AICcWt = 0.11, ΔAICc = 1.61). Overall, this suggests a lack of an effect of population of origin on body size in *H. erato*, but an effect of rearing temperature, whereby individuals reared at higher temperatures are smaller.

For *H. melpomene* pupa weight, in the RTE the highest ranked model contained both temperature and altitude as explanatory variables (without an interaction (AICcWt = 0.56, Supplementary table 2). However, when examining effect sizes and confidence intervals there only appeared to be a temperature effect, where in both populations, pupae were smaller when reared at higher temperatures, (24°C:19°C: lowland effect size = 0.80x ± 0.13, highland effect size =0.887x ± 0.102, (Figure 2D)) with no altitudinal clear effects (Supplementary table 2). For adult weight in *H. melpomene*, our highest ranked model contained an interaction between temperature and altitude (AICcWt = 0.27). Rearing temperature only had an effect on lowland populations where they became smaller at the higher rearing temperature (24°C:19°C: effect size = 0.68x ± 0.23) which was not seen in highland populations (24°C:19°C: effect size = 0.90x ± 0.21) (Figure 2B). However, this interaction effect was marginal as this model was not substantially stronger than the model without the interaction term (Delta_AICc = 0.48, AICcWt = 0.21). For both pupal and adult weights in *H. melpomene*, the highest ranked models both showed an interaction between development time with altitude, and development time with rearing temperature (pupal weight only). Therefore, the results for *H. melpomene* are similar to

H. erato, with a lack of strong evidence for population differences, but an effect of increased rearing temperature again leading to smaller body size.

Despite the lack of strong differences between populations from different altitudes, pupa and adult weights were significantly heritable traits in both *H. erato* with high ICC scores (pupa ICC =0.33, p-value = 1.24E-40; adult ICC = 0.43, p-value = 6.60E-22 (Figure 3A)) and *H. melpomene* (pupa ICC =0.18, p-value = 5.54E-10; adult ICC = 0.18, p-value = 3.25E-06 (Figure 3B)).

Survival

Neither altitudinal origin nor temperature had an effect on larval or pupal survival in either species (Figure 2). Null models were the highest ranked in both CGE (AICcWt >0.3) and RTE (AICcWt >0.7).

While larval survival was a significantly heritable trait in both *H. erato* (ICC = 0.35, p-value = 3.15E-72 (Figure 3A)) and *H. melpomene* (ICC = 0.20, p-value = 2.23E-13 (Figure 3B)) pupal survival was not significantly heritable in either species (*H. erato* ICC = 0.003, p-value = 0.4; *H. melpomene* ICC = 0.007, p-value = 0.36) (Figure 3).

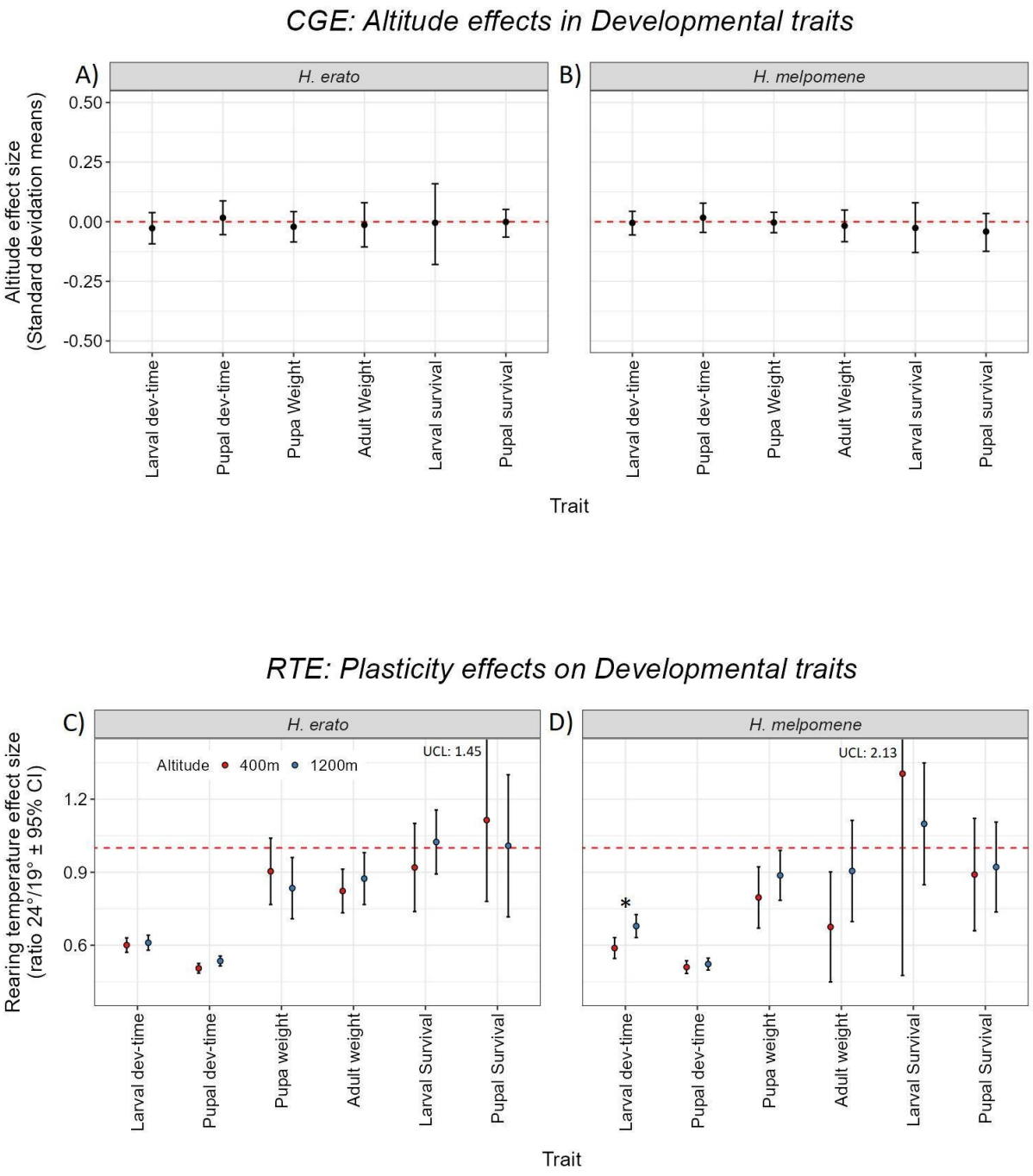


Figure 2: The effect of altitude on developmental traits in *H. erato* (A) and *H. melpomene* (B) calculated from the CGE, and the plastic effects of increased rearing temperature (as a ratio of 24°C/19°C) in highland populations (blue) and lowland populations (red) of *H. erato* (C) and *H melpomene* (D) from the RTE. Points show the mean effect size, with error bars showing the 95% confidence intervals, and asterisk denoting substantial GXE

interaction between temperature and altitudinal population. Upper confidence limits (UCL) are given for traits where this extends off the y-axis.

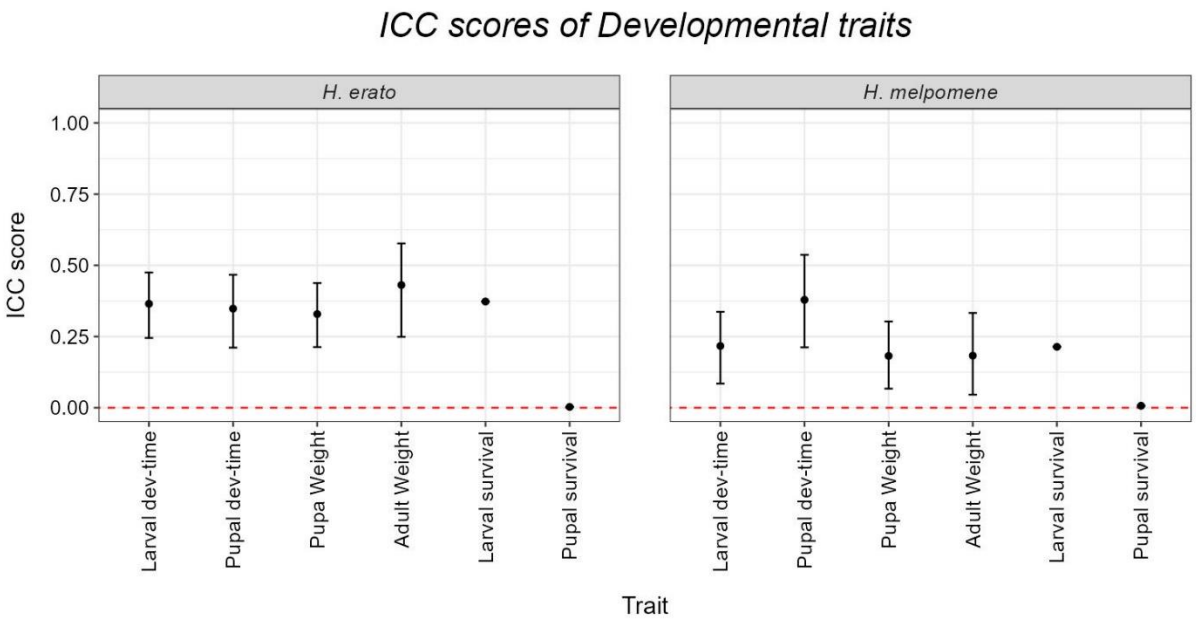


Figure 3: ICC scores of developmental traits. Points give the ICC scores and errors bars the 95% confidence intervals. All developmental traits were significantly heritable, except for pupal survival in both *H. erato* (A) and *H. melpomene* (B).

Wing Morphologies

Wing loading

The GCE showed no evidence for differences in wing loading across altitudes in either species (Figure 4). The null model was the highest ranked model in both *H. erato* (AICcWt =0.47) and *H. melpomene* (AICcWt =0.4) in explaining wing loading. In the RTE, temperature had no effect on wing loading at either altitude in *H. erato* (Figure C) and the highest ranked model contained development time alone (AICcWt =0.19), where longer development times led to the greater wing loading (smaller wings relative to body weight. In *H. melpomene*, there was a possible effect with higher temperatures resulting in greater wing loading in individuals from both altitudes although the 95% CIs cross one (Figure

4D). The highest ranked model included temperature and elevation as well as interactions between altitude with, development time and sex (AICcWt =0.13).

Wing load was a significantly heritable trait in both *H. erato* (ICC = 0.44, p-value = 5.68E-25) and in *H. melpomene* (ICC = 0.39, p-value = 5.28E-11).

Aspect ratio

As reported in Montejo-Kovacevich *et al.*, (2019), which analysed the same CGE samples we use here, wings became rounder (lower aspect ratio) with increasing altitude in both *H. erato* and *H. melpomene*. Conversely, in our RTE, altitude alone was not a relevant factor in either species (likely due to the smaller sample size in the RTE) nor was there an effect of temperature (Figure 4C and 4D)). For *H. erato* the null model was the preferred model in explaining wing aspect ratio (AICcWt =0.1, ΔAICc = 1.38), as the top model, which contained sex alone (AICcWt = 0.19) was within 2 AIC units. Whereas *H. melpomene*, the highest ranked model for wing aspect ratio showed a correlation with altitude when in interaction with sex (AICcWt =0.15). Aspect ratio was previously shown to be heritable in Montejo-Kovacevich *et al.*, (2019).

Black Pigmentation

In the CGE, altitude was shown to be a relevant factor in explaining the variation in the percentage of black on the wing (hereafter "black wing percentage") in both *H. erato* and *H. melpomene.* Where altitude increased, black wing percentage also increased (Figure 4A and 4B) with preferred models for both species having altitude an explanatory factor (*H. erato* AICcWt = 0.14; *H. melpomene* AICcWt = 0.1). However, in the RTE, altitude was only found to be a relevant factor when interacting with temperature in *H. erato*, where altitude differences were only found when populations were reared at 24°C where they were darker at higher altitudes (400m:1300m = 0.95x ± 0.03). This supported our

findings in the CGE, but there was no difference in black wing percentage when reared at 19°C (400m:1300m = 0.98x ± 0.03). Our highest ranked model supported this interaction (AICcWt =0.19) and also suggested correlation with temperature when interacting with sex, where females had greater black percentage at higher rearing temperatures in comparison to males, which reversed at lower rearing temperatures. Therefore, there was evidence of an altitudinal association with black wing percentage as well as an interaction effect with temperature in *H. erato*.

In *H. melpomene*, in the RTE, altitude showed no relevance in explaining variation in black percentage (see supplementary Figure 3B) and temperature only showed an effect when interacting with sex and adult weight but not alone (Figure 4D) (AICcWt =0.14). Males were larger than females at higher rearing temperatures, which was reversed at colder temperatures. Larger body sizes tended to have greater black percentage at higher rearing temperatures, while smaller body sizes had greater black percentage at lower rearing temperatures. Therefore, while there was an altitudinal and temperature effect on black percentage in *H. melpomene*, these factors did not interact with one another.

Unsurprisingly given the significant population effects observed, black wing percentage was a significantly heritable trait in both *H. erato* (ICC = 0.5, p-value = 1.26E-32 (Figure 5A)) and *H. melpomene* (ICC = 0.36, p-value = 3.57E-22 (Figure 5B)).

Red patch reflectance

In *H. erato*, altitude showed no substantial effect on red wing patch reflectance (Figure 4A)) with the highest ranked model containing sex as a relevant explanatory factor (AICcWt = 0.34). However, in *H. melpomene*, the highest ranked model showed that

altitude was a relevant factor but only when interacting with sex (AICcWt = 0.75) and not alone (Figure 4B). *H melpomene* females showed less reflectance with increasing altitudes, while males relatively stayed the same regardless of altitude. Variation in red reflectance could be explained substantially by sex in both species. Red reflectance was not measured in the RTE due to excessive mechanical damage in the red patch associated with non-experimental effects.

Red reflectance was a significantly heritable trait in both *H. erato* (ICC = 0.26, p-value = 1.67E-14, Figure 5A) and *H. melpomene* (ICC = 0.13, p-value = 6.58E-08, Figure 5B).

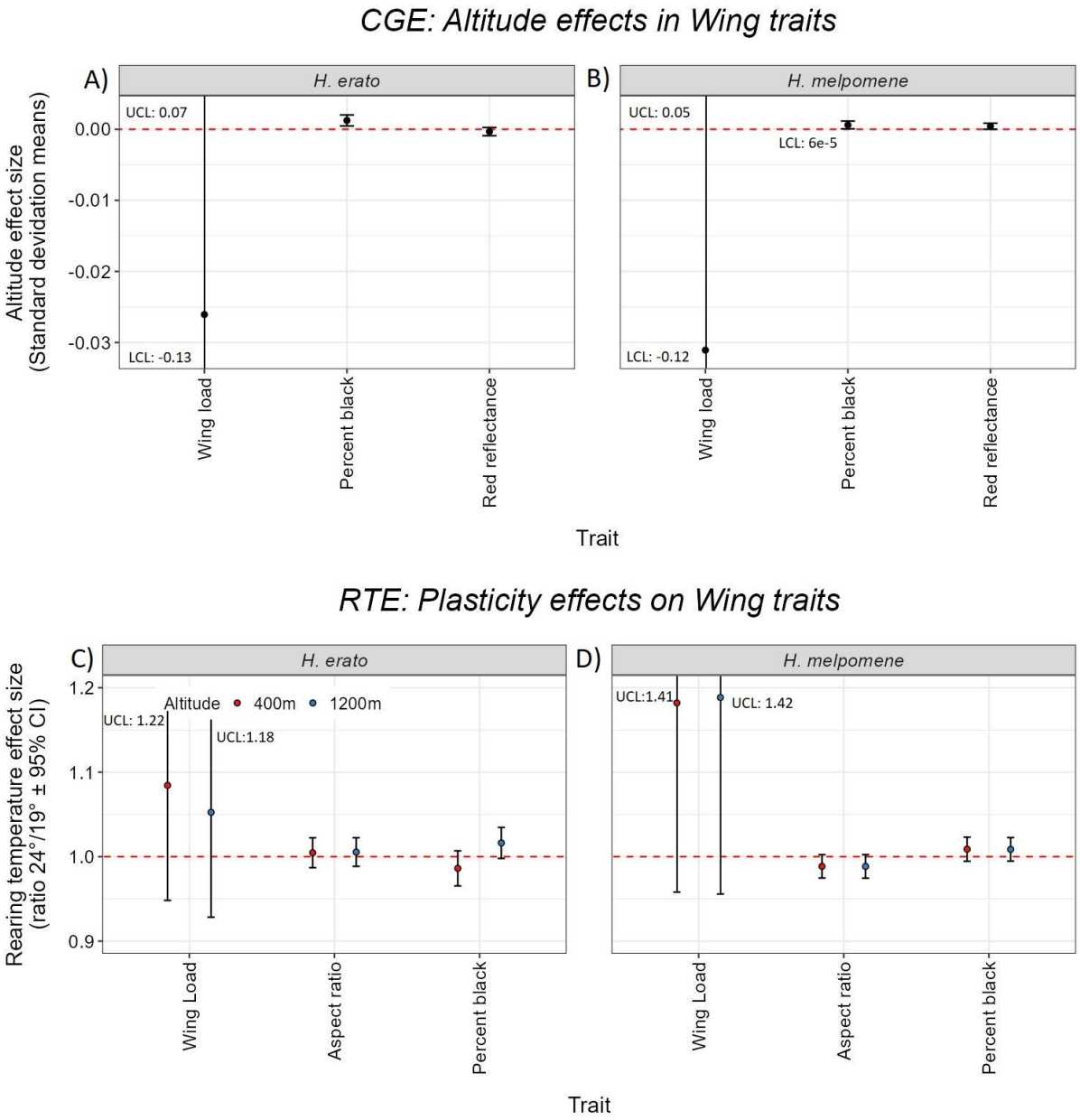


Figure 4 : The effect of altitude on wing morphological traits in *H. erato* (A) and *H. melpomene* (B) calculated from the CGE, and the plastic effects of increased rearing temperature (as a ratio of 24°C/19°C) in highland populations (blue) and lowland populations (red) of *H. erato* (C) and *H melpomene* (D) from the RTE. Points show the mean effect size, with error bars showing the 95% confidence intervals. Upper confidence limits (UCL) are given for traits where this extends off the y-axis.

Figure 5: ICC scores of wing morphological traits with 95% confidence intervals showing all measured traits were significantly heritable in both *H. erato* (A) and *H. melpomene* (B)

Thermal tolerance

Cold tolerance

In the CGE, for time to chill coma, the highest ranking model included altitude, (*H. erato* AICcWT = 0.14; *H. melpomene* AICcWT = 0.07), but altitude did not have a substantial effect in explaining variation in that trait (Figure 6). Rather, in both species body size explained the majority of the variation in time to chill coma, where increased body size led to individuals taking longer to reach chill coma. In *H. erato*, altitude had no effect on cold recovery time (Figure 6A) and the highest ranking model showed that black wing percentage alone was the strongest explanatory factor (AICcWT = 0.6), but this effect was not substantial. In *H. melpomene*, even though altitude was in the top ranking model for recovery time (along with black percentage, aspect ratio and body weight (AICcWT = 0.6))

it did not have a substantial effect (Figure 6B). Only body size had a substantial effect on cold recovery, where increased body sizes led to decreased recovery times.

In the RTE in *H. erato,* there appeared to be an interaction between rearing temperature and altitude, where only in highland populations did time to chill coma decrease with increased rearing temperature (24°C:19°C: 0.70x ±0.22), which was not otherwise seen in the lowland populations (24°C:19°C = 0.81x ±0.21) (Figure 6C). However, this interaction effect was marginal, as the highest ranked model had temperature as the sole explanatory factor explaining time to chill coma in *H. erato* (AICcWT = 0.51). Therefore, there was no altitudinal association with cold tolerance in *H. erato* however there was an effect of rearing temperature.

In *H. melpomene*, there appeared to be an interaction between altitude and temperature where at 19°C, the time to chill coma decreased with increasing altitude (400m/1200m = 0.40x ± 0.31) which was not seen at 24°C (400m/1200m = 0.88x ± 0.6) (See Supplementary Figure 4B). This interaction effect was marginal as the highest ranking model for time to chill coma was the null model (AICcWT = 0.5). Rearing temperature also had no effect on cold recovery time, with the null model as the highest ranking model in both *H. erato* (AICcWT = 0.47 (Figure 6C)) and *H. melpomene* (AICcWT = 0.28 (Figure 6D)). Therefore, there was neither an effect of altitude nor temperature on cold tolerance in *H. melpomene*.

Time to chill coma in *H. erato* was found not to be a significantly heritable trait (ICC = 0.033, p-value = 0.2 (Figure 7A)), whereas in *H. melpomene* it was (ICC = 0.12, p-value = 1.89E-03 (Figure 7B)). The inverse was then found for time to cold recovery time for each of the species, which was heritable in *H. erato* but not *H. melpomene* (*H. erato* ICC = 0.12, p-value = 5.35E-05; *H. melpomene* ICC = 0.054, p-value = 0.09) (Figure 7))

Heat tolerance

For *H. erato* in the CGE , increased altitude led to increased heat KO time and decreased time to recovery (both consistent with being more heat tolerant), which was opposite to our expectations (Figure 6A). Highest ranked models for both traits contained altitude as an explanatory factor (heat KO: AICcWT = 0.14; heat recovery AICcWT = 0.09). For heat KO, the highest ranked model also contained black percentage, and body weight, which also interacted together as pairs and with altitude. However, the model without an interaction between black percentage and body size was not 2 AICc units smaller (AICcWT = 0.08) than the highest ranked model. Increased black percentage also substantially increased heat KO time while decreasing heat recovery time, which was not what we expected. Increased body size reduced heat recovery time, but body size also had an interaction with altitude. In *H. melpomene* while altitude was a factor in the highest ranked models for both heat KO time (AICcWT = 0.07), and heat recovery (AICcWT = 0.04), altitude did not show a substantial effect (Figure 6B). Only body size showed a substantial effect on *H. melpomene* heat KO time, where individuals that were larger as pupae had increased heat KO time.

In the RTE, heat KO time in *H. erato* showed a substantial interaction between rearing temperature and altitude (AICcWT = 0.67), where increased rearing temperatures increased heat KO time in lowland populations (24°C:19°C: 400m =2.13x ± 0.79) but not in highland populations (24°C:19°C: 1200m = 1.12x ± 0.37) (Figure 6C). In the *H. melpomene* RTE there was no effect of temperature (Figure 6D) or altitude on time to heat KO, and the null model was the highest ranking model (AICcWt =0.35).

The heritability of time to heat KO was found to be significantly heritable in both H. erato (R = 0.31, p-value = 3.90E-05), and *H. melpomene* (ICC = 0.25, p-value = 1.40E-05 (Figure 7A)). However, heat recovery was only heritable in *H. erato* (ICC = 0.26, p-value = 1.67E-03) and not *H. melpomene* (ICC = 0.04, p-value = 0.2 (Figure 7B)).

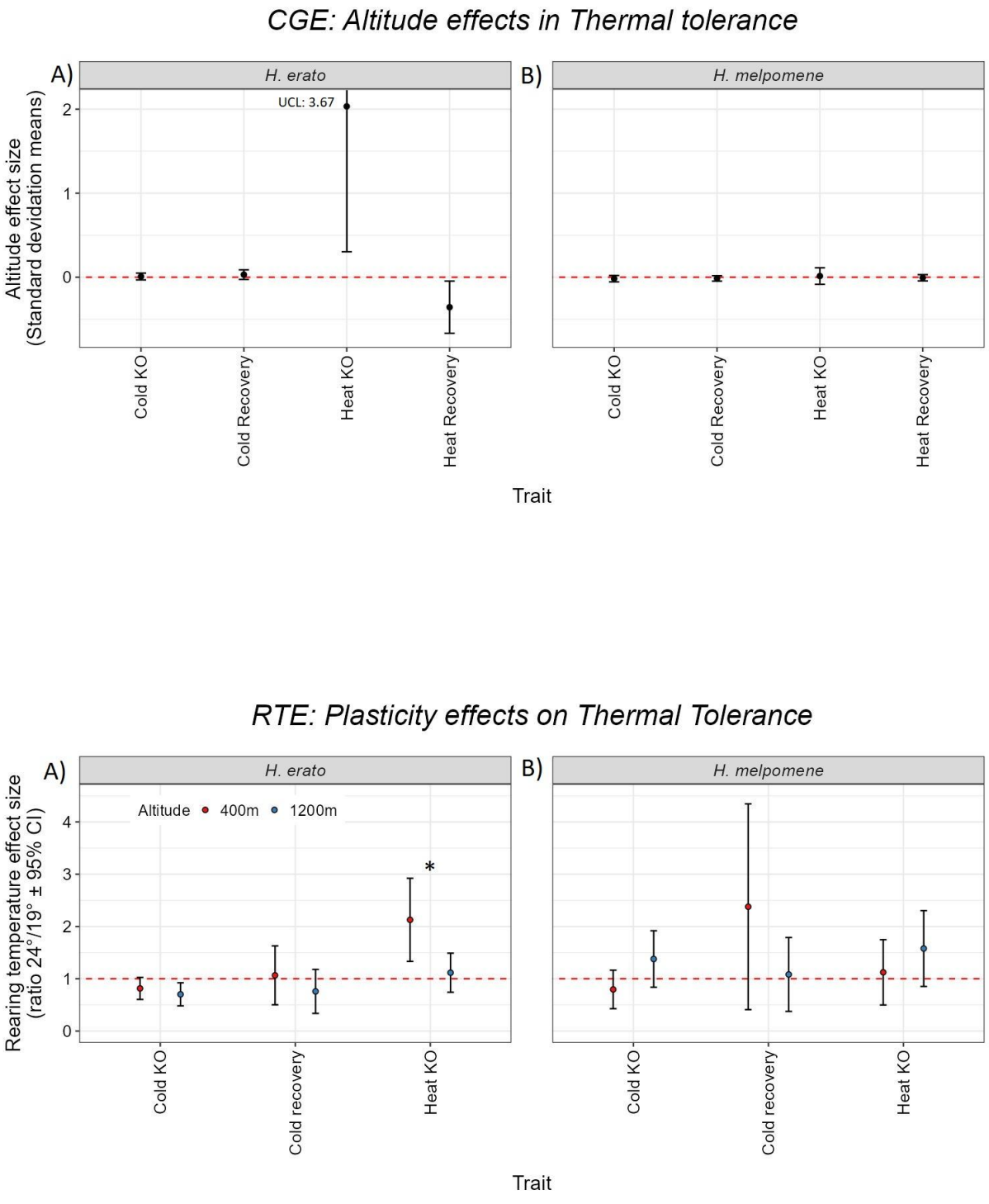


Figure 6: The effect of altitude on thermal tolerance traits in *H. erato* (A) and *H. melpomene* (B) calculated from the CGE, and the plastic effects of increased rearing

temperature (as a ratio of 24°C/19°C) in highland populations (blue) and lowland populations (red) of *H. erato* (C) and *H melpomene* (D) from the RTE. Points show the mean effect size, with error bars showing the 95% confidence intervals, and asterisk denoting substantial GXE interaction between temperature and altitudinal population

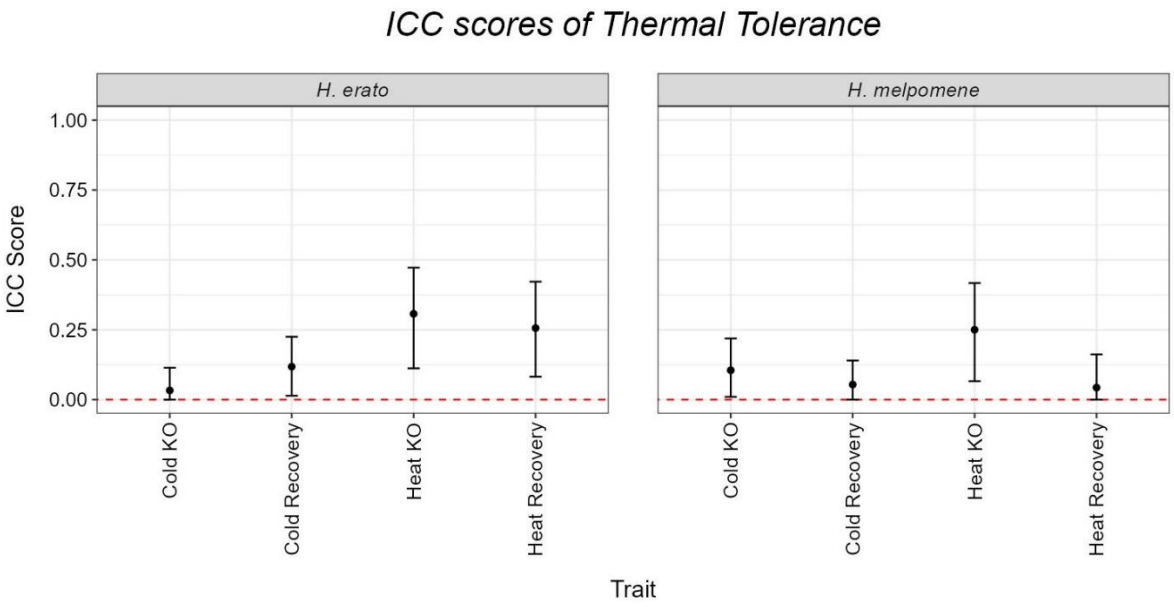


Figure 7: ICC scores with 95% confidence intervals of thermal tolerance traits *H. erato* (A) where cold KO time was not significantly heritable, but cold recovery, heat KO and heat recovery were all significantly heritable. In *H. melpomene* (B) only cold KO and heat KO were significantly heritable, whereas cold recovery and recovery were not significantly heritable.

Discussion

Altitudinal effects

The only altitudinal effects that were observed in the CGE were with regard to wing colouration and heat tolerance. In both *H. melpomene* and *H. erato* black wing pigmentation increased with altitude, while in heat tolerance, only in *H. erato* did there appear to be an effect with altitude where individuals from increasing altitude had

increased time to heat knock out, suggesting an increase in heat tolerance at higher altitudes.

Wing colour in *Heliconius* has typically been studied in the context of aposematic warnings and Müllerian mimicry, as well as sexual selection and mate choice, and the genes that underlie them have been extensively studied (The *Heliconius* Genome Consortium 2012; Nadeau, 2016). However, we have found evidence in both species of quantitative variation in the proportion of black pigmentation on the wing associated with altitude, evidence that environmental factors also affect wing colour in these species. This variation is also highly heritable, raising the interesting possibility that it may be controlled by some of the major colour pattern controlling genes that are known in these species, as has been found for other quantitative colour pattern variation (Bainbridge *et al.*, 2020).

The colour variation we observe is consistent with the thermal melanism hypothesis (TMH) (Clusella Trullas, van Wyk and Spotila, 2007), whereby darker individuals are found in cooler environments. However, in *H. erato* this pigmentation had the opposite effect to expectations in regard to the TMH, where increased black pigmentation increased time to heat KO while speeding up recovery, (both indicative of greater heat tolerance) but showed no effect in *H. melpomene*. The longer knockout time goes against both ecological and physical expectations as greater black pigmentation should absorb heat faster (Clusella Trullas, van Wyk and Spotila, 2007), thus in theory should lead to faster knockout times. However, it should be considered that our heat knockout conditions were artificial where in natural conditions butterflies would not just rely upon atmospheric temperature to thermoregulate but also solar radiation from the sun. Therefore, a possible explanation for the decreased heat recovery time in an ecological context could be that this increase in pigmentation, that seemingly leads to rapid heat

loss, is offset by the increased absorption of solar radiation energy which is more intense at higher elevation.

The red reflectance in *H. melpomene* was greatly influenced by an interaction between sex and altitude (which was not observed in *H. erato*). This is could be due to the behavioural differences between the two sexes, where males will traverse further distances in search of mates in comparison to females who are more strongly tied to nearby host plant abundance (Jiggins, 2017). Therefore, perhaps suggesting that females are more ecologically selected and males are more sexually selected. However, the directionality of reflectance in females is interesting as it increases with altitude, which would go against the TMH. This would not be the first example of butterflies to going against the TMH, as other Andean butterflies in the genus *Leptophobia* were found to lighten with increasing altitudes. The reason for this remained ambiguous, with suggestions of perhaps sexual selection or predation (Dufour *et al.*, 2018). This may be possible, in association with the increase of UV radiation at higher altitudes, which may enhance the red patch when more reflective thus accentuate their aposematism to birds or attractiveness to mates (Lind *et al.*, 2014; Dell'aglio, Stevens and Jiggins, 2016; McCulloch, Osorio and Briscoe, 2016; Teichmann, Thorogood and Hämäläinen, 2020).

An effect of altitude on heat tolerance was found in *H. erato* when reared in CG where it appeared that individuals sampled from higher altitude populations took longer to knock out under heat stress. This suggests that highland populations are more heat tolerant than lowland populations, which would go against expectations as well as what was observed previously by Montejo-Kovacevich *et al.*, (2020) who found no difference in heat KO time between *H. erato* populations when reared in CG. However, they only compared the two extremes rather than across altitudes as a continuous gradient and

their temperature environment was less controlled than ours was, as it was conducted outdoors. Our GC was controlled at a fairly constant rearing temperature that was colder than the lowland average but warmer than the highland average. Therefore, one possible explanation for our counterintuitive results could be a possible acclimation effect in highland populations or acclimation reversal in lowland populations (or both). For highland populations, in accordance with the beneficial acclimation hypothesis (Leroi, Bennett and Lenski, 1994) being reared at slightly higher temperatures than in their natural environment thus has primed them to withstand later extreme heat stress (Wilson and Franklin, 2002) thus giving greater heat tolerance as observed across species (Geister and Fischer, 2007; Karl *et al.*, 2008). Whereas for lowland populations as they were being reared at lower temperatures than their home environments, they may have have lost this acclimation associated plasticity thus acclimation reversal. Acclimation reversal has been observed in two species of *Ceratitis* fly where it took a matter of days in temperatures just 1.5°C lower than original heat acclimation temperatures for individuals to have heat knock-down time reduced to equal that of control samples (Weldon, Terblanche and Chown, 2011). This result is supported by our findings in the RTE where lowland populations showed greater thermal tolerance when reared in their home (hotter) environment in comparison to their lowland counterparts, and there was no difference when both populations were reared in highland (colder) environments.

These altitudinally-associated traits had significantly high heritability, which confirms that our CGE was detecting genetic differences between populations, consistent with local adaptation to altitude. However, it should be considered that we could not distinguish maternal effects from genetic effects in our experimental design, so heritability estimates are likely to be somewhat inflated, but could also be underestimated if our broods

contained half-siblings in addition to full-siblings, which although unlikely is possible (Walters *et al.*, 2012).

Interestingly, there were two traits across both species that showed differences neither with altitude nor with rearing temperature (in either population), which were mortality and wing loading. While wing loading has been associated with flight performance in higher altitudes where there is lower atmospheric pressure (Norry, Bubliy and Loeschcke, 2001) it is perhaps not unsurprising that we did not observe an association with altitude as similar results were also found across altitudinal populations of *Lycaena tityrus* (Karl *et al.*, 2008). A possible explanation is that perhaps wing aspect ratio offsets the lack of change in wing loading, which was found to change with altitude and thought to be involved with flight performance (Montejo-Kovacevich *et al.*, 2019).

The lack of differences in mortality could suggest that the daily mean temperatures we used would not cause selection during the larval or pupal stages. It is possible that temperature extremes between each altitude may be more important in causing differential selection with altitude (Ma, Ma and Pincebourde, 2021) although we admittedly did not measure larval or pupal thermal tolerance. Alternatively, temperature effects may be more important during the adult butterfly stage via an effect on lifetime reproductive success, especially as the other traits that have been observed to differ with altitude were in adult traits such as wing aspect ratio (Montejo-Kovacevich *et al.*, 2019) and wing colouration. This is a possibility as *Heliconius* are some of the longest lived species in their adult stage (Jiggins, 2017) which is also their most active stage. Temperature has been shown to affect fecundity across lepidoptera species,(Karlsson and Wiklund, 2005; Steigenga and Fischer, 2007), which we were unable to investigate

as we only looked at a single generation. It is also important to consider that could not discern between fertilised and unfertilized eggs, so did not reliably measure egg mortality. In *Drosophila* heat related survival of embryos was largely decoupled from adult thermotolerance, suggesting selection in relation to temperature may differ between life stages (Borda *et al.*, 2021).

There was no substantial effect of altitude on any of the developmental traits across both species nor with cold tolerance, yet they were significantly heritable traits. This high heritability would suggest that there is genetic variation that selection could act upon, but perhaps these traits are not selected with altitude. For example in cold tolerance, the temperatures exposures used were not necessarily field relevant across the altitudinal gradient sampled. Alternatively, selection may involve more complex interactions rather than just altitude alone, which we did later observe when considering rearing temperature.

Plasticity with temperature

In the RTE for both *H. erato* and *H. melpomene* we had observed typical patterns of the effect of rearing temperatures on developmental time, and body size, both of which decreased substantially with higher rearing temperatures. Decreases in developmental time with increases in temperature is commonly observed (Steigenga and Fischer, 2009; Fischer and Karl, 2010) and is typically associated with increased metabolic rates (Nedvěd, 2009). The patterns of body size followed the temperature size rule, a common reaction norm where increases in rearing temperature decrease body size (Atkinson, 1994). We found this was tightly coupled with development time, where shorter development times (caused by warmer temperatures) also led to smaller pupa. Whether

this plasticity provides an adaptive advantage or is just a general biological consequence (Kingsolver and Huey, 2008) has been contested (Teplitsky and Millien, 2014). For example, Bergmann's (1848) rule where warmer environments lead to smaller species, has often related to thermal tolerance and regulation (Horne, Hirst and Atkinson, 2018; Svensson, Gómez-Llano and Waller, 2023). However, body size has shown inverse effects across species (Mousseau, 1997; Kemp and Krockenberger, 2004; Klockmann, Günter and Fischer, 2017) including ours, where in *H. melpomene* smaller body sizes increased time to heat KO which is indicative of increased heat tolerance. Whereas, the opposite was found in *H. erato* where smaller body size decreased heat tolerance by increasing the time it took to recover from heat stress.

There did appear to be evidence of cold acclimation *H. erato*, where individuals reared at cooler temperatures took longer to enter chill coma than those reared at warmer temperatures. With respect to the beneficial acclimation hypothesis (Salt, 1961), this effect of rearing temperature on cold tolerance would be expected where the colder rearing temperature primed *H. erato* for later extreme cold exposure. This effect has been commonly observed across insect species including, *Drosophila* (Kristensen *et al.*, 2008) and other lepidopterans such as *Bicyclus anynana* (Karl *et al.*, 2014) and *D. plexippus* (Larsen and Lee, 1994). This is particularly interesting as it suggests that *H. erato* has the ability to respond to the extreme cold temperatures used in our experiments, despite the lack of altitudinal effect. The distribution of *H. erato* extends beyond the tropics in the south of America, into regions they could experience extreme cold (Rosser *et al.*, 2012) thus it is possible that there is some gene flow with the southern populations.

Chill coma recovery did not appear to show plasticity associated with temperature in either species. Rather, body size was the strongest predictor of cold tolerance in the GCE

for both species, likely because larger body sizes allow for greater buffering between organisms and the immediate environment (Bladon *et al.*, 2020).

Heat tolerance in *H. erato* was also affected by rearing temperature, where higher rearing temperatures increased the time it took for individuals to enter heat coma suggesting a plastic effect, which was also observed by Montejo-Kovacevich *et al.*, (2020) in their CG reared *H. erato*.

Gene-by-Environment interactions: Altitude and Rearing temperature

Only in larval development time in *H. melpomene*, and heat tolerance in *H. erato* showed clear and substantial gene-by-environment (GxE) interactions, with responses to rearing temperature showing evidence of local adaptation and divergence between populations.

In *H. melpomene*, the lowland larvae developed substantially faster than their highland counterparts, with increased rearing temperatures. Faster developmental times have been suggested to be beneficial to reduce vulnerability during juvenile stages to predation as well as allowing for faster dispersal rates as adults, which would be beneficial for finding mates and resources, and produce more offspring (Sinclair, Williams and Terblanche, 2012). Therefore, lowland *H. melpomene* may select for faster development times in order to compete successfully for resources, whereas in the highlands a slowed developmental time may be more beneficial, especially if resources also take longer to grow. As we observed no altitudinal differences with larval development time in the CGE, it suggests that this difference is only observed over a particular temperature threshold, where the CGE was colder than the lowland average. As the interaction between altitude and temperature was not present for pupal

development time, it could suggest that there is stronger temperature selection during larval stages than pupal in *H. melpomene*. This would be consistent if resource competition with regard to food is the selective pressure driving the difference in development time (Sinclair, Williams and Terblanche, 2012), as pupae do not eat. Furthermore, pupae stages in butterflies have been suggested to be the least sensitive to temperature (Austin and Moehring, 2019).

Heat tolerance in *H. erato* showed a substantial GXE interaction where lowland individuals, reared in their home (hotter) environment, had shown an increased heat KO time, which was not seen in highland individuals reared at the same temperatures. Therefore, lowland individuals were more heat tolerant than highland individuals when reared at higher temperatures. This suggests a degree of adaptive plasticity occurring in the lowlands that was not observed in the highlands. A possible explanation for this could be the energetic costs of maintaining acclimation ability with regard to heat shock proteins (hsp) (Feder and Hofmann, 1999). Hsps are well documented to aid in heat tolerance across species (Karlin and Brocchieri, 1998) but have high energetic costs to produce and maintain (Feder *et al.*, 1992; Krebs and Loeschcke, 1994). It has been shown that long term exposure to heat can actually be a detriment later heat tolerance, as observed in *B. anynana* (Fischer *et al.*, 2010) and the *Trichogramma carverae* wasp (Scott, Berrigan and Hoffmann, 1997). This is perhaps why we did not observe an effect of temperature in highland populations on heat tolerance. The temperature selection in the lowlands is likely greater, particularly with respect to high temperatures. Therefore, the lowland *H. erato* may have developed mechanisms to reduce or offset the cost of up-regulating hsp's during development, allowing for the acclimation and thus greater heat tolerance we observed at the constant higher rearing temperatures. This theory is

perhaps supported as the genomic region encoding hsp70 (an integral part of thermal adaptation in insects (Feder and Hofmann, 1999)) has shown evidence of altitudinal selection in the same *H. erato* subspecies (Nadeau *et al.*, 2014).

In the RTE, highland *H. erato* had substantially more black pigmentation than their lowland counterparts when reared at 24°C suggesting a possible interaction between rearing temperature as there was no difference between populations when reared at 19°C. Thermal plasticity in wing melanism has been observed to be heritable in *Pontia occidentalis* butterflies (Kingsolver and Huey, 1998).

Conclusion

In conclusion, both species showed typical patterns with rearing temperatures with particular developmental traits that are observed across Lepidoptera such as with developmental time and body size. We also discovered altitudinal differences with wing coloration in both species but only in *H. erato* did this coloration appear to play a role in thermal tolerance but in the direction that would oppose the thermal melanism hypothesis. Furthermore, only in *H. erato* did we observe an effect of altitude on heat tolerance, which also showed an interaction rearing temperature. This perhaps highlights the importance of nuance across butterfly species and their adaptations to their local environment. Our findings contribute to understanding the adaptations to and between altitude and temperature across tropical butterflies, where it is certainly not a 'one size fits all' even when occupying the same environments and niches.

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Supplementary Information

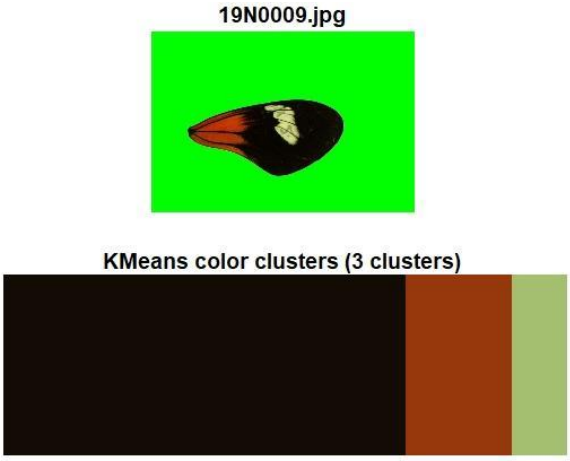
Cold Test protocol

1. Feed 5 drops again to each butterfly 1-2 hours before the heat test.
2. Butterflies are placed inside envelopes and observers are blinded to the samples origins
3. Ensure the temperature in the lab is set to 21-22°C, and record it in the notebook. This temperature is important because it can affect recovery time.
4. Ensure thermometers inside the cold test containers in the fridge read 4.5 - 5.0°C
5. Remove butterfly from the envelope and place it in the cold test container, so that it hangs facing up right from the piece of cloth glued to the chamber's wall.
6. Place cold test container in the fridge and begin the timer.
7. Record the time at which each butterfly is knocked out.
8. Shake each chamber each minute beginning at minute 3 from when it was introduced to the water, to know whether a butterfly is knocked out or not. THIS IS VERY IMPORTANT, TO RECORD 'KO TIME' ACCURATELY.
9. Remove the butterfly from the chamber exactly 10 minutes after it was introduced, using blunt forceps, and place the butterfly gently on its side in the corresponding recovery pot to the chamber it was removed from.
10. Tap each recovery pot 2-3 times with a finger or pencil each minute beginning at minute 3 from when the butterfly was placed in the recovery pot, to know whether a butterfly is recover or not. THIS IS VERY IMPORTANT, TO RECORD 'RECOVERY TIME' ACCURATELY.
11. Record the time at which each butterfly regains consciousness. This is described as point at which the butterfly is able to stand upright on all 4 of its' legs, with its thorax not touching the floor.
12. If CGE sample Feed butterfly 1 drop of food and release in it's pop-up cage again, with their original envelopes marked with rearing codes. Cover the cage with a damp cloth and a black bin liner, and leave in the laboratory. If RTE sample, preserve in RNAlater immediately after cold recovery.

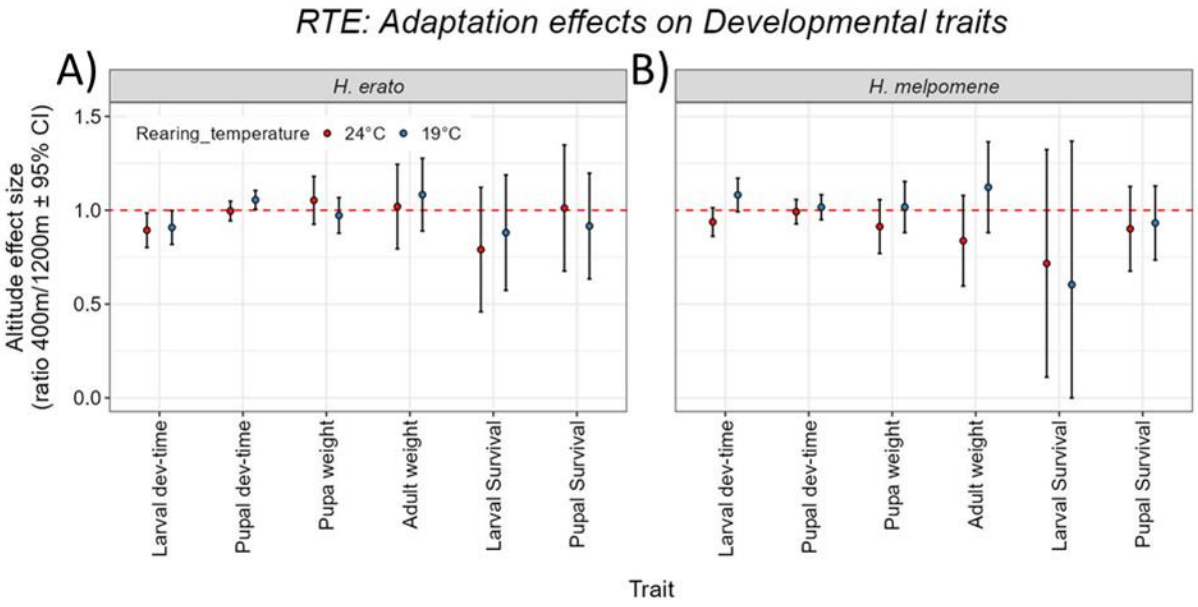
Heat test protocol

1. Feed 5 drops again to each butterfly 1-2 hours before the heat test.
2. Butterflies are placed inside envelopes and observers are blinded to the samples origins
3. Butterflies are placed in darkness until the heat tolerance test.
4. Once a heat test container inside water bath is at 40.0°C, remove it from the water bath and remove the lid.
5. Wait 1 minute before putting the butterfly in the heat test container and container into water bath
6. Record the time at which the butterfly was introduced into the water bath.
7. Record when the heat test container reaches 39.0°C.
8. Record when the chamber reaches 40.0°C.
9. The temperature within the chamber must stay within 40.0+/-1.0°C (but the aim is to keep it at 40°C). If the temperature begins to increase above 40.5°C, remove the Blu Tac on the chamber lid to open the vent to cool the chamber, or raise the lid slightly to allow cold air in, but do not remove it completely.
10. Record the time at which each butterfly is knocked out.
11. Use the thermometer to disturb the butterfly to know whether a butterfly is knocked out or not.
12. Knock out is described as the point at which the butterfly can no longer stand up on at least 3 legs; the thorax usually starts touching the floor of the chamber. A useful test is to force the butterfly onto its side with the thermometer and see if it can stand up again. If not, it is classed as knocked out.
13. Remove the butterfly from the chamber once it has knocked out. Record the time at which it knocks out, and the temperature of the chamber at that time. (In the event that a butterfly does not fall knock out two hours after the temperature inside the chamber reached 40°C – see Step 25 – the experiment should be terminated at that point, and the fact that the butterfly did not knock out within two hours of being exposed to 40°C should be recorded in the book).
14. Place the butterfly softly on its side in a recovery pot
15. Tap the pot on the lid 2-3 times gently with a finger or pencil every 5 minutes after KO.

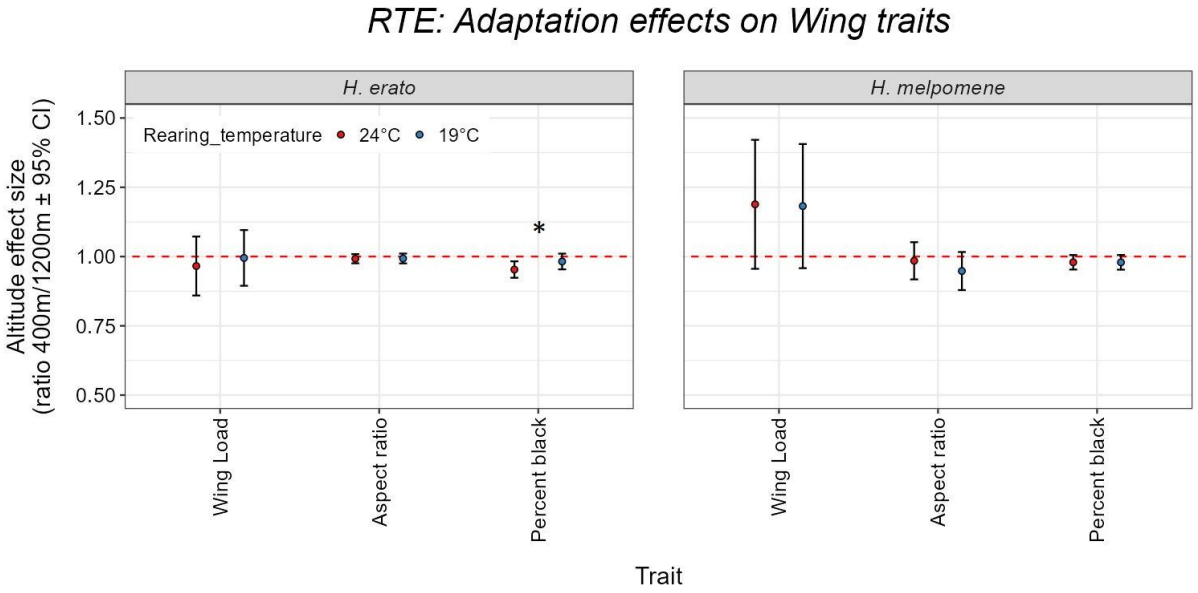
16. Record the time at which each butterfly regains consciousness. This is described as the point at which the butterfly is able to stand upright on all 4 of its' legs, with its thorax not touching the floor.
17. In the event that a butterfly does not recover fully 2 hours after it was removed from the chamber –the experiment should be terminated at this point --
18. Remove each butterfly from the recovery pot and put it back in the envelope (with the preservation code) it was taken from, and transfer the sticker from the recovery pot to the correct envelope.
19. Using the rearing code envelope, match the rearing code and the preservation code for each butterfly, and record the correct rearing code in the notebook (check that this is correct by looking at the mark on the butterfly wing)
20. The butterfly is now ready to be preserved in ethanol if GCE sample, in RNAlater if RTE sample



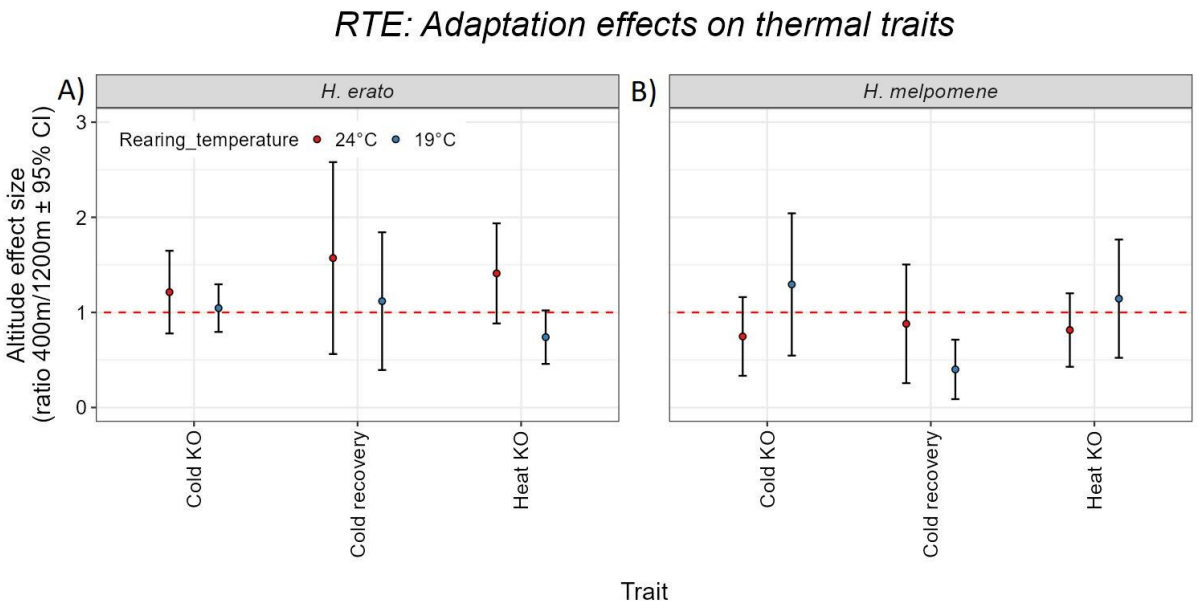
Supplementary figure 1: How package "ColourDistance" clusters together similar pixels when given an pre-defined number of clusters while ignoring pure green (background), in this case three for each forewing colour: black, red, yellow.



Supplementary figure 2: Showing the effect of altitude in *H. erato* (A) and *H. melpomene* in their developmental traits as a ratio of 400m/1200m when reared at 24°C (red) and 19°C (blue)



Supplementary figure 3: Showing the effect of altitude in *H. erato* (A) and *H. melpomene* in their wing traits as a ratio of 400m/1200m when reared at 24°C (red) and 19°C (blue), with an asterisk to denote a substantial GXE interaction between altitude and rearing temperature.



Supplementary figure 4: Showing the effect of altitude in *H. erato* (A) and *H. melpomene* in their thermal tolerance traits as a ratio of 400m/1200m when reared at 24°C (red) and 19°C (blue).

Models

Supplementary table 1: GCE models per species per trait, strongest model, model used to calculate effect size, with Delta AICc/ AICc weights, all fixed effects that were considered and the sample numbers.

species	Trait	strongest model (with fewest explanatory factors	ΔAICc	wt AICc	all fixed effects tested	Sample sizes	Effect size model	ΔAICc	wt AICc
H. erato	Laval development time	null	0	0.66	elevation	38 mothers, 775 inis	ele	1.35	0.34
H. melpomene	Laval development time	null	0	0.68	elevation	25 mothers, 450 indis	ele	1.54	0.32
H. erato	pupal development time	null	0	0.7	elevation	38 mothers 558 indis	ele	1.74	0.3
H. melpomene	pupal development time	null	0	0.7	elevation	22 mothers 405 indis	ele	1.71	0.3
H. erato	pupa weight	dev_time	0	0.47	elevation, development time	40 mothers, 753	ele + dev_time	1.58	0.21
H. melpomene	pupa weight	dev_time	0	0.67	elevation, development time	23 mothers, 444	ele + dev_time	2.02	0.24
H. erato	adult weight	dev_time	0	0.56	elevation, development time, sex	45 mothers, 327 indis	ele + dev_time	1.97	0.21
H. melpomene	adult weight	dev_time	0	0.3	elevation, development time, sex	29 MOTHER 293 INDIS	ele + dev_time	1.79	0.12
H. erato	survival to pupation	null	0	0.73	elevation	42 mothers 1549 indis	ele	2	0.27

H. melpomene	survival to pupation	null	0	0.7	elevation	26 mothers 855 indis	ele	1.74	0.3
H. erato	survival post pupation	null	0	0.73	elevation	40 mothers 802 indis	ele	2.01	0.27
H. melpomene	survival post pupation	null	0	0.6	elevation	24 mothers, 495	ele	0.77	0.4
H. erato	wing load	null	0	0.47	elevation, development time, sex	44 mothers, 319 indis	ele	1.77	0.19
H. melpomene	wing load	null	0	0.4	elevation, development time, sex	29 mothers, 290 indis	ele	1.48	0.19
H. erato	black perc	SEX + ELE + W_Area	0.5	0.14	Elevation, Sex, Wing area	36 mothers, 315 indis	same	same	same
H. melpomene	black perc	ELE + W_Area	1.63	0.1	Elevation, Sex, Wing area	23 mothers, 333 individuals	same	same	same
H. erato	red reflectance	SEX	0.53	0.34	Elevation, Sex	33 mothers, 416 indis	same	same	same
H. melpomene	red reflectance	ELE*SEX	0	0.75	Elevation, Sex	22 mothers, 431 indis	same	same	same
H. erato	CT_KO	AR + ELE + P_WEI + B.perc	0	0.14	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	37 mothers, 436 indis	same	same	same
H. melpomene	CT_KO	AR + ELE + HT_observer + P_WEI + B.perc	0	0.07	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	23 mothers, 374 indis	same	same	same

H. erato	CT_Recov	B.perc	0	0.6	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	37 mothers, 436 indis	same	same	same
H. melpomene	CT_Recov	AR + ELE + HT_observer + P_WEI + B.perc	0	0.6	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	23 mothers, 374 indis	same	same	same
H. erato	HT_KO	AR + ELE + SEX + P_WEI + B.perc + ELE:B.perc + AR:B.perc	1.18	0.08	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	31 mothers, 297 indis	same	same	same
H. melpomene	HT_KO	AR + ELE + HT_observer + P_WEI + B.perc	0.37	0.07	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	20 mothers, 239 indis	same	same	same
H. erato	HT_Recov	AR + ELE + HT_observer + P_WEI + B.perc + ELE:P_WEI	0	0.09	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	31 mothers, 297 indis	same	same	same
H. melpomene	HT_Recov	AR + ELE + HT_observer + P_WEI + B.perc	0.81	0.04	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	20 mothers, 239 indis	same	same	same

Supplementary table 2: RTE models per species per trait, strongest model, model used to calculate effect size, with Delta AICc/ AICc weights, all fixed effects that were considered and the sample numbers.

species	Trait	strongest model (with fewest explanatory factors	ΔAICc	wt AICc	all fixed effects	n numbers	Selected model	ΔAICc	wt AICc
H. erato	larval development time	TEM	1.44	0.26	Temperature, Elevation,	16 mothers, 199 indis	TEM*ELE	1.92	0.2
H. melpomene	larval development time	TEM*ELE	0	0.78	Temperature, Elevation,	8 mothers, 79	same	same	same
H. erato	Pupal development time	TEM	1.77	0.15	Temperature, Elevation, Sex	15 mothers, 143 indis	TEM*ELE	0	0.35
H. melpomene	Pupal development time	TEM	0	0.46	Temperature, Elevation, Sex	8 mothers, 68	TEM*ELE	3.99	0.06
H. erato	pupal weight	TEM*Dev_time	0	0.46	Temperature, Elevation, developmental time	16 indis, 196	TEM, ELE +DEV_TIME+ TEM:ELE+ TEM:DEV_TIME	1.82	0.18
H. melpomene	pupal weight	TEM, ELE, DEV_TIME, TEM:DEV_TIME, ELE:DEV_TIME	0	0.56	Temperature, Elevation, developmental time	8 mothers, 75 indis	TEM+ELE+ DEV_TIME+ TEM:ELE+ TEM:DEV_TIME+ ELE:DEV_TIME	1.34	0.29
H. erato	adult weight	TEM	1.61	0.11	Temperature, Elevation, developmental time, Sex	15 mothers, 131 indis	TEM, ELE, DEV_TIME, TEM:ELE, TEM:DEV_TIME	3.41	0.05
H. melpomene	adult weight	ELE:DEV_TIME +ELE +DEV_TIME	0.48	0.21	Temperature, Elevation,	8 mothers, 58	TEM+ELE+ DEV_TIME+ TEM:ELE+	0	0.27

					developmental time, sex		TEM:DEV_TIME+ ELE:DEV_TIME		
H. erato	larval survival	null	0	0.46		16 mothers, 297 indis	TEM*ELE	4.25	0.06
H. melpomene	larval survival	null	0	0.37		9 mothers, 155 indis	TEM*ELE	3.2	0.08
H. erato	pupal survival	null	0	0.54		16 mothers, 200 indis	TEM*ELE	5.66	0.03
H. melpomene	pupal survival	null	0	0.38		9 mothers, 116 indis	TEM*ELE	3.72	0.06
H. erato	Wing load mg/mm	Dev_time	0	0.19	Temperature, elevation, sex, development time	14 mothers, 127 indis	TEM:ELE +Dev_time + ELE + TEM	4.68	0.02
H. melpomene	Wing load mg/mm	ELE:Dev_time + ELE:SEX + TEM +ELE + SEX +Dev_time	0	0.13	Temperature, elevation, sex, development time	8 mothers, 87 indis	ELE:Dev_time + ELE:SEX + ELE:TEM + TEM +ELE + SEX +Dev_time	1.58	0.06
H. erato	black perc	TEM:ELE + TEM:SEX + TEM + ELE + SEX + W_Area + (1 | FAM)	0	0.19	Temperature, Elevation, Sex, wing area	13 mothers, 92 indviduals	same	same	same
H. melpomene	black perc	TEM:W_Area + W_area:SEX + TEM + ELE + SEX + W_area + (1 | FAM)	0	0.14	Temperature, Elevation, Sex, wing area	8 mothers, 75 indis	same	same	same
H. erato	Aspect ratio	null	1.38	0.1	Temperature, elevation, sex, adult weight	14 mothers, 127 indis	TEM*ELE	6.06	0.01
H. melpomene	Aspect ratio	ELE:SEX + SEX:A_Wei + TEM +ELE + SEX + A_Wei	0	0.15	Temperature, elevation, sex, adult weight	8 mothers, 87 indis	ELE:SEX + SEX:A_Wei + ELE:TEM+ TEM +ELE + SEX + A_Wei	9.61	0
H. erato	CT_KO	TEM	0	0.51	Temperature, Elevation, Observer	12 mothers, 35 indis	TEM*ELE	6.23	0.02

H. melpomene	CT_KO	null	0	0.5	Temperature, Elevation, Observer	6 mothers, 23 indis	TEM*ELE	4.19	0.03
H. erato	CT_Recov	null	0	0.47	Temperature, Elevation, Observer	12 mothers, 35 indis	TEM*ELE	3.95	0.07
H. melpomene	CT_Recov	NULL	0	0.28	Temperature, Elevation, Observer	6 mothers, 23 indis	TEM*ELE	6.12	0.02
H. erato	HT_KO	TEM*ELE + Observer	0	0.67	Temperature, Elevation, Observer	12 mothers, 45 indis	same	same	same
H. melpomene	HT_KO	null	0	0.35	Temperature, Elevation, Observer	6 mothers, 35 indis	TEM*ELE	same	same

Heat tolerance: Time to heat coma results with non-knockouts

Models:

Experiment	**species**	**Trait**	**strongest model (with fewest explanatory factors**	**ΔAICc**	**wt AICc**	**all fixed effects**	**n numbers**
CGE	*H. erato*	HT_KO	AR + ELE + SEX + P_WEI + B.perc + ELE:B.perc	0	0.15	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	31 mothers, 367 indis
CGE	*H. melpomene*	HT_KO	AR + ELE + HT_observer + P_WEI + B.perc + ELE:SEX	0	0.06	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	20 mothers, 275 indis
CGE	*H. erato*	HT_Recov	AR + ELE + HT_observer + P_WEI + B.perc + ELE:P_WEI	0	0.06	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	31 mothers, 278 indis
CGE	*H. melpomene*	HT_Recov	AR + ELE + HT_observer + B.perc	0	0.1	Elevation, Observer, Black wing percentage, pupa weight, sex, wing aspect ratio	20 mothers, 228 indis
RTE	*H. erato*	HT_KO	TEM*ELE + Observer	0	0.93	Temperature, Elevation, Observer	12 mothers, 46 indis

Chapter 4
Local Adaptation and Plasticity in gene expression responses to thermal stress in tropical butterfly populations from different altitudes

Tien T. T. Nguyen[1], Patricio A. Salazar[2], Kimberly G. Gavilanes[3], Michelle Guachamín[3], Caroline N. Bacquet[3], and Nicola J. Nadeau[1]*

[1]School of Biosciences, University of Sheffield, Alfred Denny Building, Western bank, Sheffield S10 2TN, UK

[2]Wellcome Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Saffron Walden CB10 1RQ

[3]Universidad Regional Amazónica IKIAM, Kilómetro 7 Vía Muyuna, Ciudad de Tena, 150102, Tena, Napo, Ecuador

*Corresponding author

Email: n.nadeau@sheffield.ac.uk

Author contributions

This chapter was written in manuscript format for future submission. Myself, Nicola Nadeau and Patricio Salazar designed the research project. Capture, rearing and thermal tolerance testing were conducted by Patricio A. Salazar, Kimberly G. Gavilanes, Michelle Guachamín in Ecuador. I extracted all the RNA for all samples. I analysed the transcriptomic data. I wrote the manuscript with feedback from Nicola Nadeau, who also contributed to data interpretation.

Abstract

Temperature is a fundamental ecological driver in insect adaptation, and the ability to mediate variations in temperature can determine survival. The tropics are thought to be

particularly vulnerable to climate change with rising temperatures, as species are suggested to currently live dangerously close to their thermal maximums. *Heliconius erato* are widespread neotropical butterflies which can be found along the altitude slope of the Andes. Altitudinal clines offer natural temperature gradients, allowing a fantastic environment to investigate how temperature shapes intraspecific variation and the mechanisms that underpin thermal tolerance, including acclimation. Through a transcriptomic approach, we demonstrate local adaptation and plasticity between wild populations of *Heliconius erato* from the altitudinal extremes of their range that were reared in their reciprocal temperature environment and exposed to extreme thermal stress post eclosion. Differential expression between rearing temperatures and altitudes do not appear to overlap, showing separate plastic and genetic effects on gene expression. However, when considered together in response to heat stress, we found evidence for gene-by-environment interactions, where lowland individuals reared in their home (warmer) environment exposed to heat stress had up-regulated genes with enriched GO terms associated with heat response, which were not otherwise observed at colder rearing temperatures or in highland populations. Furthermore, some heat shock proteins up-regulated in lowland populations reared in their home (warmer) temperature when exposed to heat stress, were also up-regulated in highland populations reared in their home (colder) temperatures when exposed to cold stress. This divergence between populations in their gene expression response to thermal stress, suggests opposing selection pressures between populations. We also identified genes unique to *H. erato* in response to heat stress, which may suggest unique adaptational mechanisms that are worth further investigation and pose positive future climate change implications, as these mechanisms appear to allow lowland *H. erato* to better withstand heat stress.

Introduction

Temperature is a fundamental ecological driving force that shapes the life history of organisms, in particular insects, in regard to their behaviour, physiology, and distribution (Huey and Kingsolver, 1989; Kellermann and van Heerwaarden, 2019). The ability to tolerate variations in temperature can ultimately determine survival. The onset of climate change, has created an era of rapidly changing environments centred around temperature heterogeneity with increasing averages, magnitude of variations, and also the frequency of extreme events (Halsch *et al.*, 2021). Understanding how insects adapt and respond to temperatures will hold greater relevance than ever before. We focus on the tropics, as due to the lack of seasonality organisms are thought to be less adapted to temperature variation (Janzen, 1967), and to currently live closely to their thermal maximums(García-Robledo *et al.*, 2016). Therefore, it is thought that tropical species are some of the most vulnerable to future climate change, yet have received little attention (Hodkinson, 2005; Slade and Ong, 2023).

In thermal stress response, heat shock proteins (hsp) are often involved, and have been thoroughly researched, perhaps because they are highly conserved across species, being found in mammals to insects (Karlin and Brocchieri, 1998). They act as chaperones to prevent and reverse protein denaturation and aggregation during extreme heat and are regularly reported to up-regulate during high heat exposure (Krebs and Bettencourt, 1999; Zhao and Jones, 2012). However, hsp expression is energetically costly which may compete with general housekeeping metabolism, and even increase vulnerability to other stressors(Feder *et al.*, 1992; Krebs and Loeschcke, 1994). Furthermore, a rising concern has been that not much else is understood about heat response, aside from the role of hsps. This has posed the question of whether there is a constraint with heat adaptations

(González-Tokman *et al.*, 2020). If hsps are the only physiological mechanism to tolerate high heat exposure, which cannot otherwise be maintained for prologued periods without potentially fitness costs (Feder *et al.*, 1992; Krebs and Loeschcke, 1994; Silbermann and Tatar, 2000), this may pose a detriment in future climate scenarios.

Cold response may appear to be less relevant in tropical systems, but temperature fluctuations still occur in the tropics even without seasonality (Bonebrake and Deutsch, 2012) and low temperatures are found at high altitudes in the tropics. An overlap between heat and cold adaptations have also been shown. For example, Rinehart *et al* (2007) showed there was an up-regulation of heat shock proteins during diapause in multiple *lepidoptera,* which increase cold hardiness, including in tropical moth *manduca sexta*. Furthermore, cold acclimations have shown to aid in survival in extreme heat exposure(Hemmati *et al.*, no date; Colinet *et al.*, 2013). Therefore, it is important to consider both ends of thermal adaptation, as they may overlap.

Acclimation is a common adaptation that is associated with temperature response, in which mild exposure to temperature stress can aid in survival when exposed to extremes later on (Wilson and Franklin, 2002). Using proteomics, heat acclimation has been linked to chromatin remodelling and epigenetics in *Drosophila* (Colinet *et al.*, 2013) both of which can affect gene expression and regulation. Therefore, may be why we observe an increase in hsp expression during heat stress of heat acclimated insects (Alattal and Alghamdi, 2023; Yang *et al.*, 2023). In *Bicyclus anynana,* a transcriptomic study revealed an association between acclimation to antioxidant markers thought to be linked to increased oxidative stress during heat stress (Franke *et al.*, 2019).

Environmental gradients, provide an excellent mechanism into understanding how species have evolved and adapted to change (Hodkinson, 2005). Altitude, in particular,

provides a temperature gradient which allows us to investigate temperature responses (Körner, 2007) across populations to determine whether they are a result of plasticity, genetic adaptation or a combination of both (Fox *et al.*, 2019). This natural temperature gradient also simulates potential future climate scenarios with rising temperatures, therefore allows us to predict and understand the adaptive capacity of species, and provide insights into their ability to survive and persist in altered environments (Berg *et al.*, 2010).

There have been numerous studies which have observed various adaptations and differences of butterflies along altitudinal gradients, including increased melanisation of bodies and wings which aid in solar protection and thermoregulation (Kingsolver and Buckley, 2017). In addition to the up-regulation of hsp70 expression in lowland populations of *Lycaena tityrus* (temperate species) at higher rearing temperatures (Karl *et al.*, 2009). However, these studies often provide little information on the underlying genetic mechanisms that create and maintain these responses.

Heliconius butterflies boast numerous genomic resources (The Heliconius Genome Consortium 2012) with habitat ranges across the neotropics (Jiggins, 2017) yet little is known about their thermal adaptations. Previous studies have found evidence of altitudinal adaptations in regard to wing shape (Montejo-Kovacevich *et al.*, 2019; Montejo-Kovacevich *et al.*, 2021) with some plastic variation in heat tolerance(Montejo-Kovacevich *et al.*, 2020). In particular, *Heliconius erato* has shown evidence of possible altitudinal selection on the locus region containing hsp70 (Montejo-Kovacevich *et al.*, 2021, 2022). Thus, here we used wild populations of *H. erato* from two altitudinal populations, allowing the potential to uncover local adaptations regarding gene expression in response to temperature stress and rearing. This transcriptomic approach

allows us to bridge the gap between phenotypic output and gene expression, unravelling the roles of plasticity and genetic adaptation on a molecular level to explain temperature responses. We therefore hypothesize that heat shock proteins may be differentially expressed between altitudes possibly dependent upon rearing temperature. In addition, we also hypothesize that the number of differentially expressed genes are reflective of environmental condition in traits that are particularly plastic. Furthermore, this investigation also holds the potential to uncover new genes or further our knowledge into poorly understood mechanisms and pathways of thermal adaptation in wild tropical populations, and to assess the adaptive capacity of these populations in the face of changing environments, offering valuable insights into species' resilience and survival strategies under shifting climatic conditions.

Methods

Samples

Samples were from the offspring of wild-caught females of *H. erato lativitta*, from two altitudes approximately 400 ± 50m (representing our lowland population) and 1200 ± 100m above sea level (representing our highland population). The offspring from both altitudes were reared in growth chambers under two temperature treatments: 19°C and 24°C, representing the average conditions in the forest understory of highland and lowland altitudes respectively (Montejo-Kovacevich *et al.*, 2020). Once the offspring reached adult butterfly stage, they were tested for their tolerance to thermal extremes of hot (39°C (± 1°C) until heat coma) or cold (5 ± 1 °C for 10 minutes and then recovery from chill coma) and immediately preserved in RNAlater. Full details on specimens associated with capture, rearing and thermal tolerance testing can be found in chapter 3.

Sample sizes for each rearing condition, rearing temperature, altitude and thermal stress can be found in Table 1. For full information on samples per family and sex, see Supplementary table 1 we aimed to obtain one sample of each sex for each family from each treatment combination, to ensure that factors such as family and sex are balanced across the treatments. QIAGEN RNAeasy mini kits were used to extract RNA following the manufacturer's protocol and included the additional QIAGEN DNAase step to remove any additional DNA contamination (QIAGEN, no date b, no date a). RNA concentration and quality were assessed via nanodrop (260/280 and 260:230 > 1.8), qubit, and the Agilent RNA 6000 nano kit procedures (total concentration (ng) > 20 - 1000 ng in 10-50 μl). All extracted RNA samples were stored at -80°C plated in the 96 well plate and shipped with dry ice.

Rearing temperature		19°C		24°C	
	Altitude	High	Low	High	Low
Test	Cold	9	6	5	10
	Heat	10	7	10	9
	Control	10	8	7	10

Table 1: Sample size numbers by rearing temperature, altitude and thermal test.

Sequencing

Library preparation, sequencing, trimming and quality control were completed by Liverpool centre for genomic research. Library preparation was completed using NEBNext polyA selection and Ultra II directional RNA library preparation kits. Sequencing was conducted with one lane illumina NovaSeq using S4 chemistry. This was paired-end sequencing 2x150 bp, generating an estimated 2000 million clusters per lane.

The raw Fastq files are trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1 (Martin, 2011). The option '-O 3' was used, to trim every 3' end of all reads that matched the adapter sequence >3 bp. The reads were further trimmed using Sickle version 1.200 with a minimum window quality score of 20. Reads shorter than 15 bp post trimming were removed.

Statistics were generated using fastq-stats from EAUtils.

Sequence alignment

HISAT2 was used to align sequences against the *H. erato* genome (Lepbase, 2016). Stringtie was used to assemble and quantify transcripts, with the stringtie 'merge' option to allow novel genes to be included against the existing *H. erato* gene annotation (Lepbase, 2017). Packages "IsoformSwitchAnalyzeR" and "stringr" were used to retrieve the gene names and was conducted in R Vers 4.0.0 within Rstudio.

Analysis

All analysis was completed using R Vers 4.0.0 and graphs were generated using 'ggplot'.

Differential expression

Differential expression (DE) was analysed using Bioconductor package 'EdgeR' (Robinson, McCarthy and Smyth, 2009). Filtering and normalisation of gene counts were conducted with Generalized linear model functionality. A single model incorporating altitude, rearing temperature and thermal test was used to allow for multifactor and interaction term comparisons in accordance with the EdgeR manual to find DE genes and estimate dispersion (Chen, Y., McCarthy, D., Ritchie, M., Robinson, M., Smyth, G., & Hall, 2020).

Gene set enrichment analysis (GSEA)

A gene set enrichment analysis (GSEA) was conducted to determine if any functional category of genes were significantly enriched in any of the sets of DE genes identified. First, to identify the functional categories that genes belonged to, a BLASTx using the *H. erato* protein sequences for the entire RNAseq gene count matrix, was run against the UniProt *Drosophila melongaster* proteome (UniProt, no date a), with e-value of <0.01 for homology level hits. Once gene names of the *D. melanogaster* equivalent were obtained, DE gene lists were run through DAVID (Database for Annotation, Visualization and Integrated Discovery) an online bioinformatics resource that provides functional annotation tools to find enriched GO (gene ontology) terms (Sherman *et al.*, 2022). DAVID uses a modified Fischer exact test (EASE score) to determine enriched GO terms (Sherman *et al.*, 2022). Only GO terms with EASE scores <0.05 and Benjamini scores of <0.05 were considered to be significant. The latter accounted for multiple-testing, giving corrected false discovery rates. All gene lists were run through DAVID against a background gene list containing all the genes in the original *H. erato* gene count matrix that had BLASTx hits against *D. melanogaster*, to act as our 'genome'.

With the BLASTx we also searched for genes homologous to heat shock proteins that were DE between the contrasts, as they are well documented to be associated with temperature response (Feder and Hofmann, 1999; Krebs and Bettencourt, 1999; Sørensen, Kristensen and Loeschcke, 2003).

Results

The Biological coefficient of variation (BCV) across all samples was 0.64, which indicated that the natural variation in gene expression between RNA-seq samples was moderate.

This suggests a consistent level of biological diversity within the dataset, in which gene expression differences are likely due to biological variation Supplementary Figure 1.

Initial observations using multidimensional scaling on the entire gene count matrix (Figure 1), revealed a lack of clustering or separation on either dimension 1 (which explains up to 5% of variation) or dimension 2 (which explains up to 4% of variation) by rearing temperature, altitude, or thermal testing groups. Family groups did appear somewhat clustered. However, the samples were generally mixed (Figure 1) and there was also not any clustering by sex (Supplementary Figure 2).

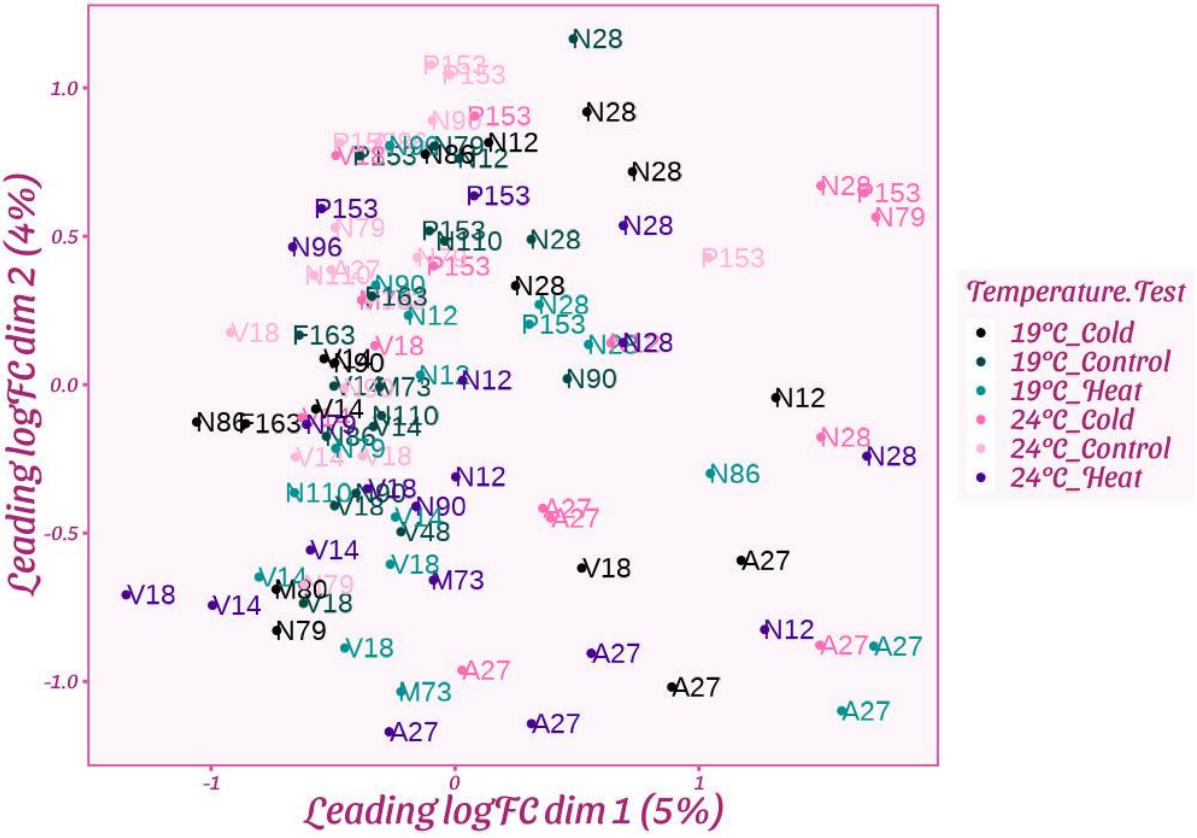


Figure 1: Multidimensional-scaling of all samples by overall gene expression. Text labels denote family group where all labels beginning with “M” and “N” are families from the highlands, while “A”, “F”, “P” and “V” are from the lowlands. Colours presenting rearing temperature and thermal tolerance test.

Single factor comparisons

When comparing only single tested factors, there was more differential expression (DE) between altitudes, with 159 up- and 154 down-regulated genes in the lowland individuals compared to the highlands, than between rearing temperatures with 66 up-regulated and 156 down-regulated in individuals reared a 24°C compared to 19°C (Table 2). This suggested a stronger population effect than plastic effect. There was little overlap in DE between altitudinal and temperature contrasts, with 1 gene that was up-regulated in both lowland relative to highland individuals and individuals reared at 24°C compared to 19°C (Figure 2), this had a *D. melanogaster* equivalent but had no gene name. There was also 1 gene that was down-regulated in lowland individuals but up-regulated in individuals reared at 24°C (Figure 2), this had no *D. melanogaster* equivalent. This lack of overlap suggests that the phenotypic and genetic differences in gene expression were distinctively separate, suggesting that any adaptive differences in gene expression between populations are not in the genes that are up or down-regulated in response to the temperatures experienced at each altitude. The distinctive difference between temperature and altitude with gene expression was also reflected in the Gene set enrichment analysis (GSEA) where the two contrasts shared no similarly enriched GO terms (Table 2).

Heat-stressed individuals had the greatest DE with 512 up- and 420 down-regulated genes when compared to their control counterparts (Table 2) and 5 of these significantly up-regulated genes had *D. melanogaster* homologues encoding for heat shock proteins (hsp): hsp68 (2 orthologous copies), Hsc70-4 and hsp83 (2 orthologous copies). However, the GSEA for DE genes in heat stressed individuals showed only 2 significantly enriched

GO terms for up-regulated genes which appeared to be non-specific to heat response and there were otherwise no enriched GO terms for the down-regulated genes (Table 2).

Cold stressed individuals had the lowest differential expression compared to their controls, with 17 up- and 6 down-regulated genes and no enriched GO terms (Table 2). Between the heat and cold stressed individuals, they shared 10 genes that were DE in the same direction in comparison to the control (7 up- and 3 down-regulated) (Table 2). These genes may be general shared stress genes or be part of the overall thermal response. Of these 10 genes, 3 had *D. melanogaster* homologues (CAH9, ORF, and cher-RF), and 6 were unannotated.

Contrast	Up- reg	Down-reg	GO terms of Up-regulated genes	GO terms of down-regulated genes
Altitude: Low vs High (controls)	159	154	5 GO:0006313~transposition, DNA-mediated (BP) GO:0060294~cilium movement involved in cell motility (BP) GO:0015074~DNA integration (BP) GO:0003693~P-element binding (MF) GO:0004803~transposase activity (MF)	3 GO:0050830~defense response to Gram-positive bacterium (BP) GO:0045087~innate immune response (BP) GO:0042834~peptidoglycan binding (MF)
Temperature: 24°C vs 19°C Controls)	66	156	none	26 GO:0022626~cytosolic ribosome (CC) GO:0002181~cytoplasmic translation (BP) GO:0005840~ribosome (CC) GO:0006412~translation (BP) GO:0003735~structural constituent of ribosome (MF) GO:0004298~threonine-type endopeptidase activity (MF)
Heat stressed vs control	512	420	2 GO:0070971~endoplasmic reticulum exit site (CC) GO:0048471~perinuclear region of cytoplasm (CC)	none
Cold stressed vs control	17	6	none	none

Table 2: Numbers of significantly DE expressed genes and related GO terms between contrasts, considering only single factors of either: altitude, temperature or thermal test. Up- and down-regulated numbers are relative to the category on the left of the contrast, and vice versa for the category on the right. For each Up- and Down-regulated gene list the two GO terms per GO term category with the lowest

Benjamini score <0.05 are included when possible (otherwise, all are presented if there were <6 total). MF = molecular function, CC - cellular component, BP = Biological process. See SI for all other enriched GO terms not in this table

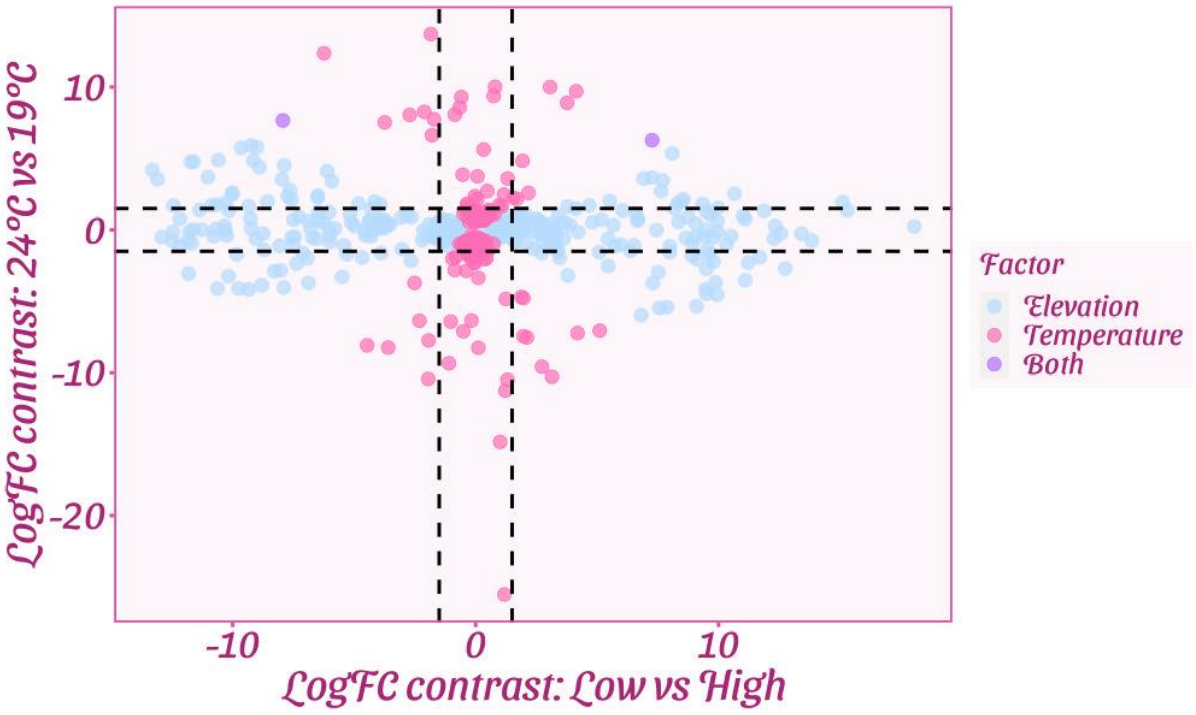


Figure 2: Significantly DE genes between individuals reared at 24°C and 19°C (y-axis), and between low altitude and high altitude populations (x-axis). The colours represent if they are significantly DE between elevations (blue), between rearing temperatures (pink) or both (purple). Negative logFC denotes a significant down-regulation of genes, of the first factor in the contrast and positive logFC denotes a significant up-regulation of genes in the first factor in the contrast.

Altitude and Rearing Temperature

When the effect of rearing temperature was assessed separately for each population (using only the control, non-stressed individuals), there was no DE between rearing temperatures in highland individuals, but there were 7 up- and 16 down-regulated genes in lowland individuals reared at 24°C versus 19°C (Table 3). When comparing populations from each altitude separately at each rearing temperature, there was no DE between altitudes when reared at 19°C, but there were 9 up- and 4 down-regulated genes

in lowland individuals compared to highland when reared at 24°C (Table 3). When testing for an interaction between altitude and temperature, there were no DE genes (Table 3).

Factor	Contrast	Up-reg	Down-reg	GO terms of up-reg genes	GO terms of down-reg genes
High altitude (controls)	24°C vs 19°C	0	0	N/A	N/A
Low altitude (controls)	24°C vs 19°C	7	16	none	none
19°C - rearing temperature (controls)	Low vs high altitude	0	0	N/A	N/A
24°C - rearing temperature (controls)	Low vs high altitude	9	4	none	none
Both: ALT*TEM High-Low (controls)	(Low.24°C - Low.19°C) - (High.24°C - High.19°C)	0	0	N/A	N/A

Table 3: numbers of significantly DE expressed genes and related GO terms between contrasts considering both altitude of origin and rearing temperature, with * denoting an interaction term between the two tested factors. Up- and down-regulated numbers denote expression direction of genes for the contrast factor on the left versus the factor on the right, except within interaction terms where up-regulated genes are for the right factor in the first bracket but left factor in the second bracket, and vice versa for down-regulated genes.

Altitude and Thermal stress

When heat stressed individuals were compared to their control counterparts separately for each population, highland individuals had fewer DE genes (70 up- and 33 down-regulated) than lowland individuals (284 up- and 178 down-regulated) (Table 4). This suggests a greater response to extreme heat in lowland individuals, which was reflected

in the GSEA, where there were more enriched GO terms in the lowland gene lists than in highland (Table 4). There were 4 significantly up-regulated genes in heat stressed individuals in both altitudes compared to their controls that had *D. melanogaster* homologues that encoded for hsps, (two hsp83 orthologs and two hsp68 orthologs). There were 4 other shared genes that appeared to have an interaction between altitude and heat stress, where in the lowlands there were 2 up- and 2 down-regulated genes that showed the opposite expression in the highlands. However, when specifically testing for an interaction between altitude and heat, only one of these genes (an unannotated gene and without *D. melanogaster* equivalent) showed significant evidence for an interaction (Supplementary table 2), but 15 other genes also showed an interaction (Table 4). Four of these significantly interacting genes were found only to be DE in the lowland comparison but not the highland comparison, while the other 12 were not found in either single test (Supplementary table 2).

For cold stress between the two populations, there were 12 (6 up- and 6 down-regulated) DE genes in the highlands and 34 (28 up- and 6 down-regulated) in the lowlands (Table 4), the two populations shared no DE genes in common. When testing for an interaction between altitude and cold stress, there were 6 up- and 7 down-regulated in lowland populations relative to highland populations (Table 4). 2 of these interacting genes were found in the other cold contrasts, 1 down-regulated in the lowland contrast and 1 up-regulated in the highland contrast (Supplementary table 3). There were no significantly enriched GO terms for DE gene lists between cold stressed contrasts for either altitude (Table 4).

Lowland populations shared 6 genes (5 up- and 1 down-regulated) between heat and cold stressed individuals. Highland populations shared 1 up-regulated gene between heat and

cold stressed individuals, which was also a gene found in the interaction contrast between altitude and cold test and was found to be significantly down-regulated in cold stressed lowland individuals

Altitude	Contrast	Up-reg	Down-reg	GO terms of up- reg genes	GO terms of down- reg genes
High	Heat test vs control	70	33	1 GO:0033180~proton-transporting V-type ATPase, V1 domain (CC)	none
	Cold Test vs Control	6	6	none	none
Low	Heat test control	284	178	7 GO:0005737~cytoplasm (CC) GO:0005524~ATP binding (MF) GO:0016887~ATPase activity (MF) GO:0003964~RNA-directed DNA polymerase activity (MF) GO:0051082~unfolded protein binding (MF) GO:0035060~brahma complex (CC)	20 GO:0031057~negative regulation of histone modification (BP) GO:0031492~nucleosomal DNA binding (MF) GO:0045910~negative regulation of DNA recombination (BP) GO:0003690~double-stranded DNA binding (MF) GO:0000785~chromatin (CC) GO:0000786~nucleosome (CC)
	Cold Test vs Control	28	6	none	none
Both: ALT*Test High-Low	(Low.Heat - Low.Control) - (High.Heat - High.Control)	8	8	none	none
	(Low.Cold - Low.Control) - (High.Cold - High.Control)	6	7	none	none

Table 4: numbers of significantly DE expressed genes and related GO terms between contrasts considering both altitude of origin and thermal test, with * denoting an interaction term between the two tested factors. Up- and down-regulated numbers denote expression direction of genes for the contrast factor on the left versus the factor on the right. The top two GO terms per GO term category were also

included when possible (otherwise, all were presented if there were <6 total) for each Up- and Down-regulated gene lists with the lowest Benjamini score <0.05. MF = molecular function, CC - cellular component, BP = Biological process. See SI for all other enriched GO terms not in this table

Temperature and thermal test

When heat stressed individuals were compared to their control counterparts for each rearing temperature separately, those reared at 19°C had more DE genes (142 up- and 42 down-regulated) than those reared at 24°C (99 up-regulated and 94 down-regulated) (Table 5). Individuals from both rearing temperatures shared 42 DE genes in the same direction (38 up- and 4 down-regulated). This suggests a similar response, with similar mechanisms being affected by heat stress, regardless of the temperature individuals were reared at. Four of the up-regulated genes found at both rearing temperatures had *D. melanogaster* homologues that encoded for hsps, (2 hsp83 orthologs and 2 hsp68 orthologs). These were the same hsp genes found up-regulated in single factor comparisons comparing heat stressed with control individuals. However, the GSEA revealed no significantly enriched GO terms in DE lists of individuals reared at 19°C, but there were 3 GO terms for 24°C. This suggests a more targeted response to heat stress occurring at higher rearing temperatures (Table 5). When testing for an interaction between rearing temperature and heat stress, there were only 2 DE genes which were down-regulated in those reared at 24°C versus 19°C, (Table 5) one of these interacting genes was found in the contrast between individuals reared at 19°C and their controls, expressed in the same direction (up-regulated) (Supplementary table 5). These two interacting genes had no *D. melanogaster* equivalent, and one was a gene that had not been previously annotated.

In cold stressed contrasts, there was only one gene DE (up-regulated) when individuals were reared at 19°C, while those reared at 24°C showed no significant DE genes between cold stressed and controls (Table 5). When testing for an interaction between rearing temperature and cold response, there were 2 up- and 8 down-regulated genes in

response to cold stress when reared at 24°C versus 19°C. One interacting gene that was significantly down-regulated in cold tested individuals reared at 24°C was one of the *D. melanogaster* hsp68 equivalents mentioned previously. Another one of the interacting genes was also found in the contrast between cold-stress and control when reared at 19°C, where it was significantly up-regulated in cold stressed individuals reared at 19°C compared to controls and cold stressed individuals reared at 24°C (Supplementary table 5). This gene had a *D. melanogaster* equivalent gene called 'rsh', predicted to be a protein sequence, but has little other information. There were no significantly enriched GO terms for DE gene lists between cold stressed contrasts for either rearing temperature (Table 5).

Rearing Temperature	**Contrast**	**Up-reg**	**Down-reg**	**GO terms of up- reg genes**	**GO terms of down-reg genes**
19°C	Heat test vs control	142	42	none	none
	Cold Test vs Control	1	0	N/A	N/A
24°C	Heat test vs control	99	94	3 GO:0004803~transposase activity (MF) GO:0003693~P-element binding (MF) GO:0005634~nucleus (CC)	none
	Cold Test vs Control	0	0	N/A	N/A
Both: TEM*Test 24°C - 19°C	(24°C.Heat-24°C.Control) - (19°C.Heat-19°C.Control)	0	2	N/A	none
	(24°C.Cold-24°C.Control) - (19°C.Cold-19°C.Control)	2	8	none	none

Table 5: numbers of significantly DE expressed genes and related GO terms between contrasts considering both rearing temperature and thermal test, with * denoting an

interaction term between the two tested factors. Up- and down-regulated numbers denote expression direction of genes for the contrast factor on the left versus the factor on the right. GO terms with Benjamini score <0.05 per GO term category were also included. MF = molecular function, CC = cellular component.

All factors

Firstly, considering highland populations only: When all parameters were considered, highland individuals when heat stressed had no DE genes compared to their controls regardless of rearing temperature (Table 6). However, there was an interaction between rearing temperature in how they responded to heat stress with 4 up- and 7 down-regulated genes in highland individuals reared at 24°C compared to 19°C (Table 6). Highland individuals reared at 24°C when cold stressed had no DE compared to their controls, but when reared at 19°C and cold stressed had 3 up- and 8 down-regulated genes (Table 6). One of these up-regulated genes had a homologous *D. melanogaster* equivalent to a hsp (hsp86), one of the same orthologs mentioned previously in heat test contrasts. There was also an interaction between rearing temperature in how they responded to cold stress with one up- and two down-regulated genes when reared at 24°C compared to 19°C. These interacting genes did not overlap with the previous highland cold stress contrasts (Table 6).

Lowland populations had more DE genes in response to heat stress (compared to controls) when reared in their home (warmer) environments at 24°C (51 up- and 16 down-regulated genes) than when reared at 19°C (26 up- and 7 down-regulated genes) (Table 6), suggesting a stronger response to heat stress when reared at 24°C. Lowland heat stress individuals shared 4 significantly up-regulated genes, regardless of rearing temperature, none with *D. melanogaster* equivalents and 3 that were previously unannotated. In the GSEA, while up-regulated genes at both rearing temperatures had

significantly enriched GO terms, only those at 24°C had temperature stress and related response terms (Table 6). This was likely because only when they were reared at 24°C were there homologous *D. melangoaster* equivalents to hsp, there were 4 in total (2 hsp68 orthologs and 2 hsp83 orthologs), the same hsp mentioned previously in heat stress contrasts, and all significantly up-regulated (Supplementary table 6). When testing for an interaction between rearing temperature and how lowland individuals respond to heat stress, there were one up- and two down-regulated genes when reared at 24°C compared to 19°C (Table 6). None of these genes were hsp's and only one of these interacting genes was found that significantly down-regulated when heat stressed reared at 24°C compared to 19°C, was also significantly up-regulated when reared 19°C and heat stressed compared to controls (Supplementary table 7). This gene had a homologous *D. melanogaster* equivalent called gag-pol, predicted to be an RNA-directed DNA polymerase (UniProt, no date b). Lowland individuals when cold stressed had more DE genes compared to their controls when reared at 24°C (8 up-regulated genes) than at 19°C (1 down-regulated gene). These genes had no significantly enriched GO terms (Table 6).

When testing for an interaction between altitude and rearing temperature and how they respond to thermal tests, there were no DE genes between altitudes when reared at 19°C in either thermal test (Table 6). When reared at 24°C there were no DE between altitudes with cold stress, but there were 2 DE (1 up- and 1 down-regulated) genes when responding to heat stress (Table 6).

Altitude	Rearing Temperature	Contrast	Up-reg	Down-reg	GO terms of up- reg genes	GO terms of down-reg genes
High	19°C	Heat stressed vs control	0	0	N/A	N/A
		Cold stressed vs Control	3	8	none	none
	24°C	Heat test vs control	0	0	N/A	N/A
		Cold stressed vs Control	0	0	N/A	N/A
	24°C - 19°C Tem*Test	(24°C.Heat-24°C.Control)-(19°C.Heat-19°C.Control)	4	7	none	none
		(24°C.Cold-24°C.Control) - (19°C.Cold-19°C.Control)	1	2	N/A	none
Low	19°C	Heat stressed vs control	26	7	9 GO:0042575~DNA polymerase complex (CC) GO:0015074~DNA integration (BP) GO:0006313~transposition, DNA-mediated (BP) GO:0003964~RNA-directed DNA polymerase activity (MF) GO:0004190~aspartic-type endopeptidase activity (MF)	
		Cold stressed vs Control	1	0	N/A	N/A
	24°C	Heat stressed control	51	16	4 GO:0051082~unfolded protein binding (MF) GO:0031072~heat shock protein binding (MF) GO:0051087~chaperone binding (MF) GO:0005737~cytoplasm (CC)	none

		Cold stressed vs Control	8	0	none	N/A
	24°C - 19°C Tem*Test	(24°C.Heat-24°C.Control) - (19°C.Heat-19°C.Control)	1	2	N/A	none
		(24°C.Cold-24°C.Control) - (19°C.Cold-19°C.Control)	0	2	N/A	none
Both: ALT*TEM High-Low	19°C	(Low.Heat - Low.Control)- (High.Heat - High.Control)	0	0	N/A	N/A
		(Low.Cold - Low.Control)- (High.Cold - High.Control)	0	0	N/A	N/A
	24°C	(Low.Heat - Low.Control)- (High.Heat - High.Control)	0	0	N/A	N/A
		(Low.Cold - Low.Control)- (High.Cold - High.Control)	1	1	N/A	N/A

Table 6: numbers of significantly DE expressed genes and related GO terms between contrasts considering all three tested factors: rearing temperature, altitude of origin and thermal test, with * denoting an interaction term. Up- and down-regulated numbers denote expression direction of genes for the contrast factor on the left versus the factor on the right. The top two GO terms per GO term category were also included when possible (otherwise, all were presented if there were <6 total) for each Up- and Down-regulated gene lists with the lowest Benjamini score <0.05. MF = molecular function, CC - cellular component, BP = Biological process. See SI for all other enriched GO terms not in this table

Discussion

Plasticity with temperature

When considering altitude and temperature as single factors across all samples, there was little to no overlap in differentially expressed (DE) or in enriched GO terms between the rearing temperature contrast and the altitudinal contrast, suggesting separate plastic and genetic effects on DE. The genes up-regulated in individuals reared at warmer temperatures showed no significantly enriched GO terms which suggests no specificity in mechanism changes in gene expression with higher rearing temperatures. While the significantly down-regulated genes in individuals reared at warmer temperatures (thus up-regulated in colder temperatures) had enriched GO terms related to mitochondrial activity, ribosomal activity and translation, which were all down-regulated at warmer rearing temperatures. The down-regulation in mitochondrial activity at higher rearing temperatures suggests a reduction in metabolic activity that would be against expectations and previous transcriptomic observations across insect species. This included three planthopper species (Huang *et al.*, 2017) and moth species *Glyphodes pyloalis* Walker(Liu *et al.*, 2017) which showed repression in metabolic activities (glycolysis, gluconeogenesis and fatty acid biosynthesis etc.) at colder temperatures

When responding to heat stress, there was a significant up-regulation of heat shock proteins (hsp) regardless of rearing temperature. The up-regulation of hsps in response to heat stress is unsurprising as it occurs across species, as they are highly conserved proteins associated with heat adaptation (Karlin and Brocchieri, 1998). There was a greater number DE genes 19°C than 24°C which initially suggested a stronger response to heat stress at lower temperatures. However, the GSEA had shown there were only enriched GO terms associated with heat response in the up-regulated genes in heated-

tested individuals reared at 24°C and not 19°C (when compared to their controls). This therefore suggested that the DE when reared at 24°C has a targeted and associated function to heat stress such as acclimation, which occurs at higher rearing temperatures as the GO terms involved transposition, which is involved in gene regulation throughout the genome (Lanciano and Cristofari, 2020).

There was little DE between cold-tested individuals and their control, regardless of rearing temperature or altitudinal origin. This was likely because the cold exposure period had only been 10 minutes, and recovery time was up to 10 minutes, (in comparison to heat stressed which lasted from 2 to 90 minutes) thus cold stresses were not long enough to elicit a change in gene expression (Bendjilali *et al.*, 2017).

Altitudinal differences

When comparing the DE genes between altitudes, the (GSEA) revealed differences in gene regulation, where there appeared to be an up-regulation in transposition activity in lowland populations but immune responses in highland populations (thus vice versa). There has been evidence of local adaptations in relation to immune response in *Lycaena tityrus* butterflies which was suggested to reflect the different environmental needs across altitudes (Karl, Hoffmann and Fischer, 2010) therefore, could be why we see differences in DE between populations of *H. erato*. However, it should be considered that immune system genes evolve much more rapidly than in other physiological systems (Lazzaro and Clark, 2012). Therefore, differences in immune responses between populations may be expected as one of the first mechanisms to diverge and, thus may not be specifically altitudinally linked.

Both lowland and highland populations responded to heat stress similarly, they shared a large proportion of DE genes including the significant up-regulation of heat shock

proteins (hsp) and even an enriched GO term related to ATP activity. As mentioned previously, heat stress response mechanisms are highly conserved across species thus the shared DE including hsp was unsurprising (Karlin and Brocchieri, 1998), and an up-regulation of ATP activity is likely associated with increased metabolism caused by the extremely high-temperature exposure (Nedvěd, 2009; Harvey *et al.*, 2020). However, lowland populations had distinctly more DE genes when compared to their controls than highland populations, some of which were found to be significantly DE between the two populations. These additional genes had also produced more enriched GO terms, suggesting this DE had specific functionality, which could be associated with heat response or stress associated with extreme heat exposure in response to heat stress. These GO terms included "unfolded protein binding" in up-regulated genes, as unfolded proteins, are typically proteins that are denatured, therefore up-regulating genes to bind to these proteins could reduce damage (Feder and Hofmann, 1999). This suggests evidence of local adaptation with gene expression in response to heat stress in lowland populations, with additional mechanisms to reduce the negative effects of constant high temperatures. Spatial gene expression differences in response to heat stress have been observed in damselfly species *Ischnura elegans* which were strongly associated with their range expansions (Lancaster *et al.*, 2016).

The enriched GO terms of up-regulated genes in highland individuals in response to heat stress had an association with DNA structure in regard to chromatin, and histone modifications. In *Drosophila*, histone modifications have been thought to be associated with epigenetic-based cold acclimation (Colinet *et al.*, 2013). This could explain why they may be up-regulated in highland *H. erato*, with inherited plasticity from the experiences of their mothers in colder environments at higher altitudes (Fox and Mousseau, 1998; Zhou *et al.*, 2013). Cold acclimation generally increases the cold tolerance, but this was

not observed in *H. erato* with no differences in cold tolerance with altitude or rearing temperature as reported in chapter 3.

Gene-by-environment interactions

When considering both altitude and temperature, highland individuals showed no DE genes between rearing temperatures, but lowland individuals did. This could suggest a gene-by-environment interaction where lowland individuals show temperature-related plasticity with gene expression, which was not observed in highland individuals. This gene-by-environment DE was further supported when both altitude and temperature were considered together in response to heat stress. Only in lowland populations when reared at 24°C (i.e their home environment) did we find up-regulated genes (including hsps), that had enriched GO terms associated with heat response that were not otherwise found in individuals reared at 19°C, or in highland populations regardless of rearing temperature. There were four hsp proteins found up-regulated with *Drosophila* homologous equivalents, they belonged to the hsp70 chaperone group (hsp68) (Feder, Blair and Figueras, 1997) and the hsp90 chaperone group (hsp83). Both complexes have been shown to be involved in heat acclimation across species including *Drosophila* (Bettencourt *et al.*, 2008; Boher *et al.*, 2012) and rice leaf roller moth *Cnaphalocrocis medinali* (Gu *et al.*, 2019). Therefore, this up-regulation and enriched GO terms suggest a degree of acclimation occurring at 24°C that is not otherwise seen at 19°C, in the lowlands. This also supports the phenotypic evidence in chapter 3 which showed a showing a gene-by-environment interaction occurring in *H. erato* where lowland individuals, when reared at warmer temperatures, were more heat tolerant in comparison to colder rearing temperatures, which was otherwise not observed in highland individuals. Temperate butterfly *Lycaena tityrus* has also shown altitudinal differences in hsp70 regulation

(which hsp68 is part of the same chaperone family (Feder, Blair and Figueras, 1997) in relation to rearing temperature in the same pattern, in which lowland populations reared at higher temperatures significantly up-regulated hsp70 levels (Karl *et al.*, 2009). Thus, even though the production of hsps is energetically costly, there is a clear selection to be able to maintain them at a high level in warmer lowland environments rather than solely during acute stress. The combination of 51 significantly up-regulated genes in lowland populations reared at 24°C may be the recipe that underlies *H. erato* heat acclimation which has been selected for in the lowlands, as the majority of the genes were distinct from the genes DE in highland populations in response to temperature. Furthermore, in this list of 51 genes, some did not have a *Drosophila* equivalent, perhaps suggesting that *Heliconius* have evolved their own unique adaptations for heat tolerance and response, which would be interesting to investigate.

Concerning hsp expression, there was one version of *H. erato* hsp68 gene that was up-regulated in lowland heat response that was also significantly up-regulated in highland populations reared in their home environments (colder) when exposed to cold stress, with the same copy significantly down-regulated under cold stress from individuals reared at higher temperatures (when altitude was not considered). Hsps are not specific to heat stress and are generally up-regulated in response to cellular stress. Furthermore, mechanisms of hot and cold stress have been shown to overlap due to some shared consequences such as desiccation (Rajamohan and Sinclair, 2008; Sinclair *et al.*, 2013) and it has been suggested that some genes have the ability to shift their function depending on thermal stress (Lancaster *et al.*, 2016). This perhaps suggests that there is an altitudinal selection of hsp68 expression, in both altitudes and for opposing responses. In the lowlands, it shows a selection for heat tolerance, while in the highlands it may show a selection for cold tolerance.

Aside from the DE of hsp68 during cold stress exposure, there were few other DE genes in response to cold, particularly when considering rearing temperature or altitude. This could suggest a lack of cold adaptation in *H. erato*, which as a tropical species may be unsurprising as temperatures are perhaps unlikely to reach their thermal minimums in the typical average temperature, even at higher elevations (Dixon *et al.*, 2009; Montejo-Kovacevich *et al.*, 2020). However, as mentioned previously, this could also be likely due to the exposure to cold stress being too short to elicit a strong, measurable response. Furthermore, 19°C may have not been low enough to trigger cold acclimation (as this was the average mean temperature of the highlands), as GSEA results did not show any specific GO terms, and there was no phenotypic evidence of cold acclimation in chapter 3 aiding cold tolerance in *H. erato*.

Conclusions

We provide evidence of local adaptation with gene expression with a gene-by-environment reaction in response to heat in *H. erato*, which suggests lowland populations are heat selected, while highland populations are possibly cold selected, but there is overall little evidence for cold adaptation. However, there were a handful of genes that did not have a *Drosophila* equivalent and even novel unannotated genes which could suggest that *H. erato* (and therefore possibly other tropical butterflies) may have unique adaptational mechanisms that are worth investigating. Furthermore, it appears that there are lowland adaptations that appear to allow the maintenance of hsp without huge detriment, which are otherwise generally known to have long term negative effects in other species (Feder *et al.*, 1992; Krebs and Loeschcke, 1994; Silbermann and Tatar, 2000). This provides positive implications for future climate change scenarios for tropical butterflies, as it suggests that *H. erato* has the potential to adapt to increasing temperatures in withstanding prolonged heat stress.

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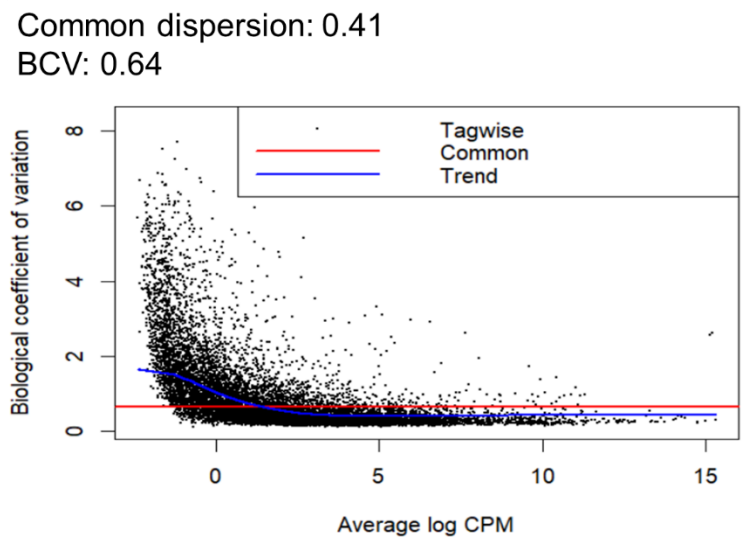
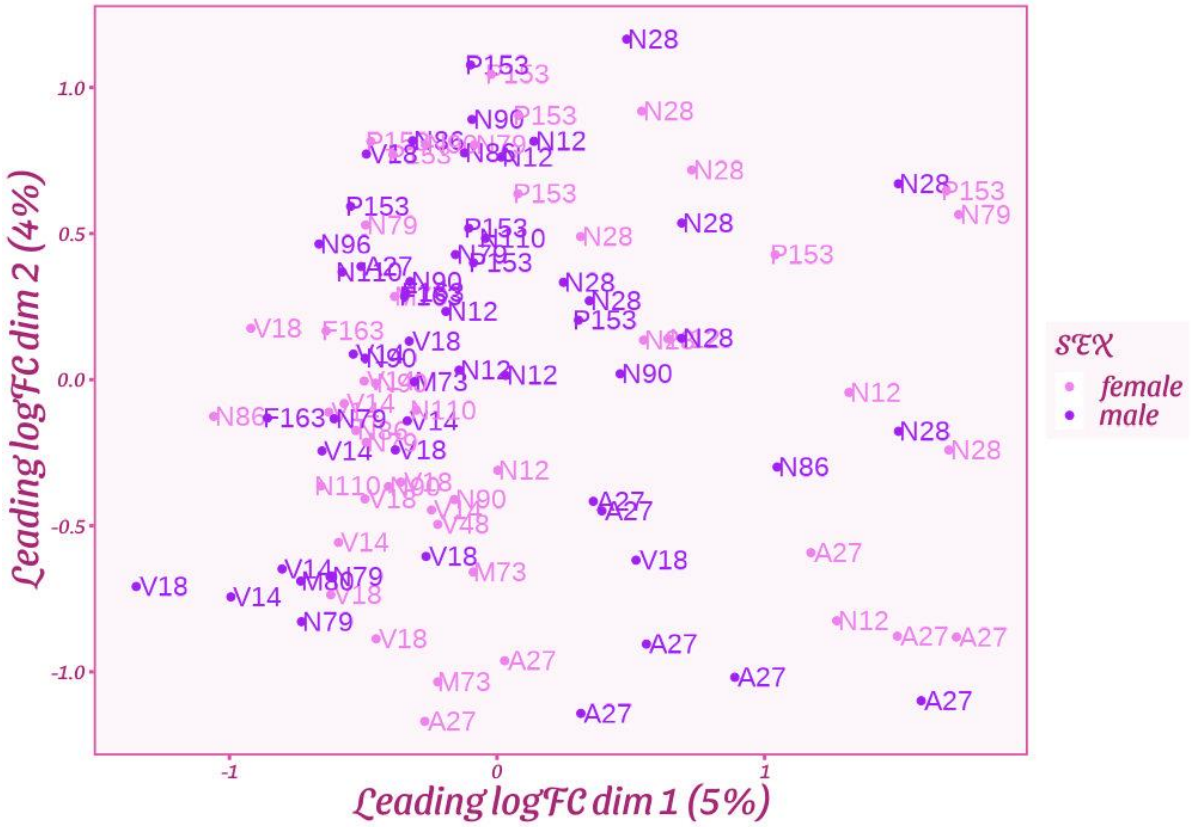
Supplementary information

Supplementary table 1: Sample size numbers by thermal test rearing temperature, sex, altitude and family.

		Cold test				**Control**				**Heat test**			
		19°C		24°C		19°C		24°C		19°C		24°C	
Elevation	**Mother**	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
High	M73			1						1		1	
	N110					1	1		1	1			
	N12	1	1	1			1				2	2	1
	N28	2	1		2	1	1			1	1	1	2
	N79		1	1		1	1	1	2	1			1
	N86	1	1			1			1		1		
	N90		1			1	1	1	1	1	1	1	
	N96												1
Totals		4	5	3	2	5	5	2	5	5	5	5	5
Low	A27	1		2	2				1	1	1	1	2
	F163		1			1	1		1				
	P153			2	1	1	1	3	1		1	1	1
	V14	1	1	1		1	1	1	1	1	1	1	1
	V18	1	1		2	2		1	1	1	1	1	1
Totals		3	3	5	5	5	3	5	5	3	4	4	5



Supplementary figure 1: A graph showing the Biological coefficient of variation (BCV) with also values of common dispersion across all samples



Supplementary Figure 2: Multidimensional-scaling of all samples by overall gene expression. Text labels denote family group where all labels beginning with "M" and "N" labels are from the highlands, while "A", "F", "P" and "V" are from the lowlands. Colours denoting sex (Pink = female, purple = male)

Differentially expressed genes

Supplementary table 2: Differentially expressed genes with their *Drosophila* homologue equivalent from the contrast containing the interaction between altitude and heat stress and the overlapping genes from other contrasts between altitude and heat stressed.

	Contrast and LogFC			
***H. erato* genes**	**High Ele: Heat vs Control**	**Low Ele: Heat vs Control**	**ELE*HeatTest: Low vs High**	**UniProtID**
MSTRG.3600	6.995493	-9.70681	-34.8257	NA
Herd.Herato0821G92	NA	NA	-21.612	Q9W1D3_DROME
MSTRG.16645	NA	NA	-19.3634	NA
MSTRG.3436	NA	NA	-18.486	NA
MSTRG.7415	NA	NA	-18.3215	NA
MSTRG.3050	NA	NA	-16.5327	NA
Herd.Herato2001G193	NA	-3.11709	-8.08367	Q9VNE8_DROME
MSTRG.14199	NA	NA	-5.70811	NA
Herd.Herato1003G103	NA	3.496247	8.104279	Q7KSE9_DROME
MSTRG.6308	NA	4.058178	8.57024	NA
MSTRG.14439	NA	4.29274	8.76835	NA
Herd.Herato2101G593	NA	NA	9.527279	NA
MSTRG.8550	NA	NA	13.14758	RTBS_DROME
MSTRG.81	NA	NA	15.47893	NA
MSTRG.16813	NA	NA	15.98081	NA
MSTRG.459	NA	NA	18.51579	NA

Supplementary table 3: Differentially expressed genes with their *Drosophila* homologue equivalent from the contrast containing the interaction between altitude and cold stressed and the overlapping genes from other contrasts between altitude and cold stressed.

	Contrast logFC			
***H. erato* genes**	**High ele: Cold vs Control**	**Low ele: Cold vs Control**	**ELE*ColdTest Low vs high**	**UniProtID**

MSTRG.3600	NA	NA	-19.9982	NA
Herd.Herato2001G193	NA	-3.01276	-8.15933	Q9VNE8_DROME
MSTRG.8550	NA	NA	16.62123	RTBS_DROME
Herd.Herato1301G389	10.76486	NA	-14.2387	Q9VD55_DROME
Herd.Herato1507G345	NA	NA	-16.7513	NA
MSTRG.2973	NA	NA	-16.3134	NA
Herd.Herato1202G388	NA	NA	-15.1793	NA
Herd.Herato0701G123	NA	NA	-14.7608	NA
Herd.Herato0419G44	NA	NA	4.533884	Q9VYJ3_DROME
Herd.Herato0401G42	NA	NA	14.45946	O96299_DROME
MSTRG.6595	NA	NA	15.23337	NA
Herd.Herato1701G345	NA	NA	15.91546	NA
MSTRG.6306	NA	NA	17.50532	NA

Supplementary table 4: Differentially expressed genes with their *Drosophila* homologue equivalent from the contrast containing the interaction between rearing temperature and heat stressed and the overlapping genes from other contrasts between rearing temperature and heat stressed.

	Contrast			
***H. erato* genes**	**Rearing temp 19°C: Heat vs Control**	**Rearing temp 24°C: Heat vs Control**	**Temp*ColdTest 24°C vs 19°C**	**UniProtID**
Herd.Herato0901G246	7.189796	NA	-17.2018	NA
MSTRG.16021	NA	NA	-14.0627	NA

Supplementary table 5: Differentially expressed genes with their *Drosophila* homologue equivalent from the contrast containing the interaction between rearing temperature and cold stressed and the overlapping genes from other contrasts between rearing temperature and cold stressed.

	Contrast		
***H. erato* genes**	**Rearing temp 19°C: Cold vs Control**	**Temp*ColdTest 24°C vs 19°C**	**UniProtID**

Herd.Herato0606G524	NA	-16.5342	HSP68_DROME
Herd.Herato0701G102	NA	-22.0934	NA
Herd.Herato0508G80	NA	-19.1068	NA
MSTRG.4082	NA	-15.7133	NA
Herd.Herato0503G184	NA	-13.7939	NA
Herd.Herato1202G748	NA	-12.4177	NA
MSTRG.8233	NA	-11.3874	NA
Herd.Herato0701G664	NA	49.10965	NA
Herd.Herato0508G27	NA	15.13963	Q8I725_DROME
Herd.Herato1603G14	5.791289	-7.75304	X2JF15_DROME

Supplementary table 6: Differentially expressed genes and their *Drosophila* homologue equivalent from between lowland individuals reared at 24°C exposed to heat stress in comparison to their controls, with their logFC to denote up- or down-regulation.

***H. erato* genes**	**Low elevation, rearing temperature 24°C: Heat vs Control logFC**	**UniProtID**
Herd.Herato1108G419	-2.15457	A0A0B4KEP0_DROME
Herd.Herato1007G209	5.481218	A0A0B4KEY5_DROME
Herd.Herato1807G31	6.775934	A0A0B4KEY5_DROME
Herd.Herato0101G263	-2.04496	A1ZAL5_DROME
Herd.Herato1507G314	1.439552	A8JNS4_DROME
Herd.Herato2101G478	1.298351	B5X0J4_DROME
Herd.Herato1003G201	-1.80735	B6V6Z8_DROME
Herd.Herato1601G97	-2.54832	B6V6Z8_DROME
Herd.Herato0801G87	-0.47543	COG2_DROME
Herd.Herato0606G524	6.221587	HSP68_DROME
Herd.Herato1805G253	4.3179	HSP68_DROME
Herd.Herato1805G249	3.835564	HSP83_DROME
Herd.Herato1805G307	3.529784	HSP83_DROME
Herd.Herato1708G103	0.547586	ILF2_DROME
Herd.Herato1007G194	2.015951	M9NCP5_DROME
Herd.Herato0204G31	0.72569	M9NEA5_DROME
Herd.Herato2001G274	0.421752	M9NEL1_DROME

Herd.Herato0411G68	0.582652	M9PBQ3_DROME
Herd.Herato0401G12	0.72589	M9PBZ0_DROME
Herd.Herato2001G107	0.424833	M9ZYW3_DROME
Herd.Herato0609G85	1.022174	MPIP_DROME
Herd.Herato0101G154	3.54149	NA
Herd.Herato0701G652	3.648749	NA
Herd.Herato0701G868	2.837158	NA
Herd.Herato1001G177	-4.69277	NA
Herd.Herato1003G300	2.710634	NA
Herd.Herato1507G114	3.098839	NA
Herd.Herato1701G345	6.130611	NA
Herd.Herato1710G6	-6.21722	NA
Herd.Herato2001G27	5.779769	NA
Herd.Herato2101G593	4.942407	NA
MSTRG.11055	6.028695	NA
MSTRG.13526	6.515798	NA
MSTRG.13535	4.470941	NA
MSTRG.1355	7.057146	NA
MSTRG.14439	4.196017	NA
MSTRG.15549	7.695038	NA
MSTRG.18126	-1.74137	NA
MSTRG.5388	7.280992	NA
MSTRG.6308	3.99339	NA
Herd.Herato1507G176	3.392106	POLR_DROME
Herd.Herato1711G3	5.493957	Q04134_DROME
Herd.Herato0101G153	3.56891	Q6NKM5_DROME
Herd.Herato1605G53	3.627277	Q6NKM5_DROME
Herd.Herato1605G57	3.602003	Q6NKM5_DROME
MSTRG.13904	3.358083	Q6NKM5_DROME
Herd.Herato0503G330	0.567242	Q6NP42_DROME
Herd.Herato0101G61	0.477055	Q7KMM4_DROME
Herd.Herato0901G218	1.256973	Q86NM8_DROME
Herd.Herato1408G156	-0.58589	Q8MQY2_DROME
MSTRG.2908	1.161798	Q8MSM5_DROME
Herd.Herato0821G95	0.740923	Q8SWZ8_DROME

Herd.Herato0211G105	-2.23287	Q8SXZ7_DROME
Herd.Herato0801G358	-6.81112	Q8SY28_DROME
Herd.Herato0209G38	2.276355	Q960B1_DROME
Herd.Herato1007G92	2.957067	Q9NAZ4_DROME
Herd.Herato1202G643	7.736903	Q9U972_DROME
Herd.Herato0606G46	1.143837	Q9VFV9_DROME
Herd.Herato0101G439	4.717208	Q9VJC6_DROME
Herd.Herato0101G638	-0.76676	Q9VM49_DROME
Herd.Herato0701G823	-0.81539	Q9VMQ6_DROME
Herd.Herato2001G193	-3.8804	Q9VNE8_DROME
Herd.Herato1904G270	1.195633	Q9VPN5_DROME
Herd.Herato0901G213	-3.44322	Q9VW98_DROME
Herd.Herato1301G356	-0.75821	ROR2_DROME
Herd.Herato1910G7	0.564418	UFD4_DROME
Herd.Herato0310G288	0.901888	X2J5E8_DROME

Supplementary table 7: Differentially expressed genes with their Drosophila homologue equivalent from the lowland contrasts containing the interaction between rearing temperature and heat stressed and the overlapping genes from other contrasts between rearing temperature and heat stressed.

	Contrast			
***H. erato* genes**	**Low ele, rearing temp 19°C: Heat vs Control**	**Low ele, rearing temp 24°C: Heat vs Control**	**Ele*Tem Low elevation: 24°C vs 19°C Heat test**	**UniProtID**
Herd.Herato0301G120	NA	NA	13.01563	NA
MSTRG.7656	6.950933	NA	-11.8631	NA
Herd.Herato0503G158	NA	NA	-19.1249	Q8MNR4_DROME

GSEA all other significant GO terms

Single factors comparisons

24°C vs 19°C – down regulated genes	
Category	Term
GOTERM_CC_DIRECT	GO:0000502~proteasome complex
GOTERM_BP_DIRECT	GO:0043161~proteasome-mediated ubiquitin-dependent protein catabolic process
GOTERM_CC_DIRECT	GO:0022627~cytosolic small ribosomal subunit
GOTERM_CC_DIRECT	GO:0005829~cytosol
GOTERM_CC_DIRECT	GO:0005839~proteasome core complex
GOTERM_BP_DIRECT	GO:0010498~proteasomal protein catabolic process
GOTERM_CC_DIRECT	GO:0022625~cytosolic large ribosomal subunit
GOTERM_BP_DIRECT	GO:0051603~proteolysis involved in cellular protein catabolic process
GOTERM_CC_DIRECT	GO:0005747~mitochondrial respiratory chain complex I
GOTERM_BP_DIRECT	GO:0006120~mitochondrial electron transport, NADH to ubiquinone
GOTERM_CC_DIRECT	GO:0005838~proteasome regulatory particle
GOTERM_CC_DIRECT	GO:0005739~mitochondrion
GOTERM_CC_DIRECT	GO:0005654~nucleoplasm
GOTERM_BP_DIRECT	GO:0032981~mitochondrial respiratory chain complex I assembly
GOTERM_CC_DIRECT	GO:0015935~small ribosomal subunit
GOTERM_CC_DIRECT	GO:0005743~mitochondrial inner membrane
GOTERM_BP_DIRECT	GO:0006511~ubiquitin-dependent protein catabolic process
GOTERM_CC_DIRECT	GO:0019774~proteasome core complex, beta-subunit complex
GOTERM_CC_DIRECT	GO:0019773~proteasome core complex, alpha-subunit complex
GOTERM_CC_DIRECT	GO:0008541~proteasome regulatory particle, lid subcomplex

Altitude and rearing temperature

High elevation: 24°C vs 19°C – down regulated genes	
Category	**Term**
GOTERM_CC_DIRECT	GO:0005829~cytosol
GOTERM_CC_DIRECT	GO:0005839~proteasome core complex
GOTERM_CC_DIRECT	GO:0008540~proteasome regulatory particle, base subcomplex
GOTERM_CC_DIRECT	GO:0005654~nucleoplasm
GOTERM_BP_DIRECT	GO:0051603~proteolysis involved in cellular protein catabolic process
GOTERM_CC_DIRECT	GO:0019773~proteasome core complex, alpha-subunit complex
GOTERM_BP_DIRECT	GO:0006511~ubiquitin-dependent protein catabolic process

GOTERM_CC_DIRECT	GO:0008541~proteasome regulatory particle, lid subcomplex
GOTERM_CC_DIRECT	GO:0022626~cytosolic ribosome
GOTERM_CC_DIRECT	GO:0005840~ribosome
GOTERM_CC_DIRECT	GO:0022627~cytosolic small ribosomal subunit
GOTERM_MF_DIRECT	GO:0070628~proteasome binding
GOTERM_BP_DIRECT	GO:0002181~cytoplasmic translation
GOTERM_CC_DIRECT	GO:0005743~mitochondrial inner membrane
GOTERM_CC_DIRECT	GO:0019774~proteasome core complex, beta-subunit complex
GOTERM_BP_DIRECT	GO:0030163~protein catabolic process
GOTERM_MF_DIRECT	GO:0003735~structural constituent of ribosome
GOTERM_BP_DIRECT	GO:0006412~translation
GOTERM_MF_DIRECT	GO:0004843~thiol-dependent ubiquitin-specific protease activity
GOTERM_BP_DIRECT	GO:0016579~protein deubiquitination
GOTERM_CC_DIRECT	GO:0005737~cytoplasm

Low elevation: 24°C vs 19°C – down regulated genes	
Category	Term
GOTERM_CC_DIRECT	GO:0022626~cytosolic ribosome
GOTERM_CC_DIRECT	GO:0005743~mitochondrial inner membrane
GOTERM_CC_DIRECT	GO:0005747~mitochondrial respiratory chain complex I
GOTERM_CC_DIRECT	GO:0000502~proteasome complex
GOTERM_CC_DIRECT	GO:0005838~proteasome regulatory particle
GOTERM_BP_DIRECT	GO:0006099~tricarboxylic acid cycle
GOTERM_CC_DIRECT	GO:0022627~cytosolic small ribosomal subunit
GOTERM_BP_DIRECT	GO:0043161~proteasome-mediated ubiquitin-dependent protein catabolic process
GOTERM_CC_DIRECT	GO:0005759~mitochondrial matrix
GOTERM_CC_DIRECT	GO:0008540~proteasome regulatory particle, base subcomplex
GOTERM_CC_DIRECT	GO:0031430~M band
GOTERM_CC_DIRECT	GO:0005829~cytosol
GOTERM_CC_DIRECT	GO:0022625~cytosolic large ribosomal subunit
GOTERM_CC_DIRECT	GO:0030018~Z disc
GOTERM_CC_DIRECT	GO:0005839~proteasome core complex

Altitude and thermal stress

Low elevation: heat test vs control – down regulated genes

Category	Term
GOTERM_BP_DIRECT	GO:0031057~negative regulation of histone modification
GOTERM_MF_DIRECT	GO:0031492~nucleosomal DNA binding
GOTERM_BP_DIRECT	GO:0045910~negative regulation of DNA recombination
GOTERM_BP_DIRECT	GO:0016584~nucleosome positioning
GOTERM_BP_DIRECT	GO:0051276~chromosome organization
GOTERM_MF_DIRECT	GO:0003690~double-stranded DNA binding
GOTERM_MF_DIRECT	GO:0031490~chromatin DNA binding
GOTERM_BP_DIRECT	GO:0030261~chromosome condensation
GOTERM_CC_DIRECT	GO:0000785~chromatin
GOTERM_CC_DIRECT	GO:0000786~nucleosome
GOTERM_BP_DIRECT	GO:0031507~heterochromatin assembly
GOTERM_MF_DIRECT	GO:0030527~structural constituent of chromatin
GOTERM_BP_DIRECT	GO:0006334~nucleosome assembly
GOTERM_MF_DIRECT	GO:0003677~DNA binding
GOTERM_BP_DIRECT	GO:0015074~DNA integration
GOTERM_CC_DIRECT	GO:0005634~nucleus
GOTERM_BP_DIRECT	GO:0006313~transposition, DNA-mediated
GOTERM_MF_DIRECT	GO:0003693~P-element binding
GOTERM_MF_DIRECT	GO:0004803~transposase activity
GOTERM_MF_DIRECT	GO:0004190~aspartic-type endopeptidase activity
GOTERM_CC_DIRECT	GO:0042575~DNA polymerase complex

Chapter 5

General Discussion

In this thesis, I have presented *Heliconius* as an excellent neotropical model and highlighted the importance of a holistic approach to investigating thermal adaptation, linking phenotype to genotype with gene expression. Through a combination of rearing experiments of both non-wild and altitudinal populations, coupled with transcriptomics, this thesis has begun to uncover the genetic basis, with gene expression, of thermal tolerance and temperature response across *Heliconius*. Below, I provide a summary of the main findings and integrate possible future directions for further research and investigations.

Phenotypic responses to temperature and altitude

Across all three species (*Heliconius sara, Heliconius erato* and *Heliconius melpomene*), we observed typical patterns where higher rearing temperatures decreased developmental time and consequently led to small body sizes, which aligned with the temperature-size response often observed in insects (Atkinson, 1994). However, in *H. sara*, developmental time had an interaction with rearing temperature in adult weight. While pupae were larger at colder rearing temperatures (as larvae had more time to feed and accumulate resources), during metamorphosis longer developmental time at colder temperatures resulted in smaller adults. This is possibly associated with optimal developmental temperatures (Kingsolver *et al.*, 2007; Steigenga and Fischer, 2009) where *H. sara* pupae were experiencing a stronger degree of stress at colder temperatures, leading to a greater depletion of energy stores resulting in smaller adults post eclosion. This shows the importance of considering each stage within the life cycle, which may reveal unexpected patterns, perhaps indicating environmental suitability. The interaction between

developmental time and temperature was not observed in *H. erato* and *H. melpomene* perhaps because their rearing temperatures had been the average daily means at both altitudes, whereas for *H. sara* the temperatures, while field relevant, were more extreme(Montejo-Kovacevich *et al.*, 2020) and therefore, resulted in a more prominent effect on body size. Furthermore, it should be noted that in *H. melpomene*, the effect of temperature had a gene-by-environment interaction (GXE) in larval development time, where lowland larvae developed significantly faster in warmer (which was also their home environment) temperatures than highland individuals. This suggests that body size was a trade-off with development time, as in their home environments faster development may be advantageous, allowing for faster dispersal rates and reduced vulnerability in immobile juvenile stages (Sinclair, Williams and Terblanche, 2012). The body size and developmental time trade-off was perhaps further supported as larger body sizes were observed to increase both heat and cold tolerance in *H. melpomene* (and *H. erato*). Larger body sizes having greater advantages against thermal extremes may also be why we did not observe altitudinal variation with body size in either mimetic species as both populations would perhaps experience equal (hot or cold) extremes.

The effect of temperature on thermal tolerance was interesting as it differed across all three species. In *H. sara*, rearing temperatures reflected tolerance to both cold and heat, in *H. erato* warmer rearing temperatures increased heat tolerance (specifically in lowland populations), while rearing temperature did not affect *H. melpomene* thermal tolerance whatsoever. The lack of acclimation occurring in *H. melpomene* heat tests may be because rearing temperatures were not high enough to trigger the acclimation and hardening response. The rearing temperatures in the reciprocal transplant experiment (RTE) were daily mean temperatures and therefore had no acclimation response in *H. melpomene*.

However, what was particularly interesting was the evidence of local adaptation in *H. erato*, where there was a GXE in heat tolerance between rearing temperature and altitude, where warmer rearing temperatures led to higher heat tolerance only in lowland and not in highland individuals (or in *H. melpomene*). This suggests that lowland *H. erato* have unique adaptations to their local environment that provide an advantage in withstanding extreme heat that was not otherwise observed in *H. melpomene* even though they occupy similar niches. In the context of climate change, this may suggest differing responses between the mimetic species.

Notably, our investigations revealed quantitative variations in the proportion of black pigmentation on the wings, linked to altitude in both *H. erato* and *H. melpomene* where with increasing altitudes, butterflies had a greater proportion of black pigmentation on their wings. Therefore, providing evidence that environmental factors play a role in influencing wing colouration in these species. Initially, this colouration was thought to have an adaptive purpose in thermoregulation, as colouration patterns were in line with the thermal melanism hypothesis (TMH) (Clusella Trullas, van Wyk and Spotila, 2007). However, black pigmentation did not affect thermal tolerance in *H. melpomene,* while in *H. erato* increased black wing pigmentation increased heat tolerance by decreasing heat coma recovery, which was against our expectations. In an ecological context, this could be because this increase in pigmentation, which seemingly leads to rapid heat loss, is offset by the increased absorption of solar radiation energy that is more intense at higher elevations, or functions and an adaptation under natural basking and flight conditions, which we were unable to test. With this interesting observation of colouration, altitude and thermal tolerance in *H. erato,* a possible future avenue for investigation is, the role of iridescence in *H. sara*. While *H. sara* is a heat-hardy species (Montejo-Kovacevich *et al.*,

2020) rearing temperatures appear to have little effect on their tolerance against extreme heat, thus they may rely on other mechanisms to thermoregulate, such as with basking behaviours, in which their iridescence may play a role. It would be interesting to compare thermoregulatory capacity between iridescent and non-iridescent species or with other mimicking species with the same iridescence or ones with a combination of pigment and iridescence, such as *Heliconius doris* (Parnell *et al.*, 2018). *H. sara* scales produce black pigment, but their cuticle nanostructures create a blue iridescence through the bending of light(Parnell *et al.*, 2018) and therefore, energy. The thermoregulatory role of iridescence is an understudied area which could provide fascinating results. Bosi *et al* (2008) found that iridescent species of *lepidoptera* had greater absorbance compared to non-iridescent species, while noting a distinct lack of iridescent species in temperate regions. While Bosi *et al* (2008) had observed no correlation between habitat temperature and solar wing absorption they had only examined 64 species total, across both temperate and tropical regions, therefore, it would be difficult to exclude the possible thermoregulatory role of iridescence in tropical regions without considering additional factors such as microhabitat selection. For example, Douglas *et al* (2007) correlated iridescence with habitat type, with forest rather than open environments in neotropical nymphalid butterflies, therefore the greater solar absorption in lower light environments may aid in thermoregulation.

The only traits that showed variation across altitudes were heat tolerance in *H. erato* and black wing pigmentation in both *H. erato* and *H. melpomene*. Both of these traits were also heritable traits which could further be investigated through a Genome-Wide Association Study as we had SNPs genotyped the DNA from individuals from the 'common garden' experiment in chapter 3. This would allow us to potentially identify the genetic basis of

the altitudinally-linked traits (as conducted previously by Montejo-Kovacevich *et al* (2021) with wing aspect ratio), and understand their genetic architecture, and mechanisms of adaptation to altitude changes. Understanding the genetic foundation of altitude-related traits holds substantial implications for conservation efforts, particularly in climate change. This understanding aids in predicting population responses to shifting altitudinal conditions and in identifying crucial candidate genes governing these traits. Additionally, it provides insights into the underlying mechanisms controlling these traits and offers a deeper comprehension of their complexity

Transcriptomic responses to temperature and altitude

Temperature had a strong influence on gene expression in both *H. sara* and *H. erato.* The differentially expressed (DE) genes when comparing rearing temperatures across both species shared a few enriched GO terms involved with mitochondrial activity, ribosomal activity and translation, which were all down-regulated at warmer rearing temperatures. The down-regulation in mitochondrial activity at higher rearing temperatures suggests a reduction in metabolic activity, which appears to be a unique temperature response in our *Heliconius* species as it goes against expectations and previous transcriptomic observations across other insect species. Three planthopper species (Huang *et al.*, 2017) as well as moth species *Glyphodes pyloalis* Walker (Liu *et al.*, 2017) showed repression in metabolic activities (glycolysis, gluconeogenesis and fatty acid biosynthesis etc.) at colder temperatures. However, the differences in gene expression between developmental stages between rearing temperatures in *H. sara* followed similar patterns to what has been observed in flesh fly, *Sarcophaga similis* (Harada and Goto, 2017), in fruit fly *Bactrocera tau* (Huang *et al.*, 2020) and in *D. melanogaster* (Austin and Moehring, 2019). This may suggest differing mechanisms of thermal response and adaptation in each

developmental stage, which should therefore be considered separately in investigations. We did not have pupae or larval samples in *H. erato* to compare, but it would be interesting to observe the effect of rearing temperature on altitudinal populations of larvae and pupae of *H. erato* (and *H. melpomene*), and perhaps even embryonic egg stages to observe whether altitude affects gene expression.

In both *H. sara* and *H. erato* rearing temperature had an effect on thermal tolerance, suggesting acclimation was occurring. When cold stressed *H. sara* were compared to their controls, they had no DE genes, this was likely due to the length of cold-stress exposure. However, there was a substantial difference in recovery time between rearing temperatures, this suggests that baseline gene expression at colder-rearing temperatures has allowed for a cold hardening effect. Therefore, the broad non-specific enriched GO terms between rearing temperatures may suggest that cold acclimation may involve broad preventative measures that require further investigations to understand their underlying mechanisms within tropical systems.

H. sara did not show evidence of heat acclimation associated with rearing temperature, whereas *H. erato* showed a GXE in heat tolerance mentioned previously, which was then reflected in their gene expression, with a significant up-regulation of heat shock proteins and enriched go terms associated with heat response, protein folding and refolding. This same upregulation of heat shock proteins and enriched GO terms was also observed in the heat-tolerant individuals of *H. sara*, and therefore suggests that heat adaptation mechanisms are more corrective than preventative. While heat shock proteins are chaperones that do bind to proteins to prevent denaturing, they are energetically costly to produce constantly (Feder *et al.*, 1992; Krebs and Loeschcke, 1994). Therefore, heat shock proteins are only induced when required to prevent further denaturing, and can

perform their corrective roles in folding and refolding denatured proteins caused by high temperatures (Krebs and Bettencourt, 1999; Zhao and Jones, 2012).

The reason why we observe an up-regulation of heat shock response proteins in heat-tolerant individuals of *H. sara* and lowland individuals of *H. erato* is perhaps that these individuals have mechanisms that affect the induction speed (Sejerkilde, Sørensen and Loeschcke, 2003) and concentration of heat shock proteins. *Lycaena tityru*s, has even shown similar patterns in heat tolerance with altitude, but hsp70 expression was greater in lowland populations than in highlands linked to greater heat tolerance (Karl *et al.*, 2009). This is perhaps contrary to *Drosophila* studies, where lower concentrations supposedly indicate stronger heat tolerance (Sørensen, Dahlgaard and Loeschcke, 2001; Sørensen *et al.*, 2005). This emphasizes the importance of understanding heat response and how it may suggest differing mechanisms of thermal adaptations across species. Therefore, an intriguing avenue for exploration would involve RNA interference (RNAi) experiments to downregulate the expression of these heat shock proteins in *Heliconius* species (in particular the *Drosophila* homologue equivalents of Hsp68 and Hsp83), enabling us to observe their response

From the reciprocal transplant experiment detailed in Chapter 3, we conducted RNA expression sequencing on *H. melpomene* individuals. Comparing the RNAseq analysis of *H. erato* in Chapter 4 with that of *H. melpomene* could unveil conserved pathways of thermal adaptation across altitudes and potentially reveal species-specific adaptations influencing the observed phenotypic differences in thermal responses between the two species detailed in Chapter 3. Additionally, our RNA expression sequencing of *H. melpomene* unexpectedly led to the discovery of a *Heliconius timerata* population—a cryptic species closely related to *H. melpomene* (Nadeau *et al.*, 2014). This would allow us

to perform a comparative RNA expression analysis across *H. sara* (Chapter 2), *H. erato, H. melpomene*, and *H. timerata* offering an opportunity to unravel the intricate molecular mechanisms governing thermal response across the *Heliconius* genus with comprehensive understanding. Examining RNA expression patterns would allow us to outline shared pathways indicative of conserved responses to thermal challenges and discern species-specific adaptations that have evolved. This investigation holds the potential to shed light on the nuanced interplay between conserved and specialized responses, enriching our understanding of the evolutionary dynamics and ecological diversification within *Heliconius*.

Due to the nature of our experiments, we were unable to account for multi-generational responses, which have been previously observed in rice leaf folder moth *Cnaphalocrocis medinali* in heat acclimation with regard to heat shock protein expression (Gu *et al.*, 2019). Nor were we able to observe the effect of rearing temperature on fecundity. Therefore, it would be interesting to observe whether the altitudinal differences and GXE interactions are consistent after multiple generations, or how fast temperature selection would occur.

Conclusion

In conclusion, in this thesis, I have shown that *Heliconius* show altitudinal variation with wing colouration (in *H. erato* and *H. melpomene*) and heat tolerance (*H. erato* only). There was also evidence of local adaptation in plasticity with a GXE interaction with higher rearing temperatures leading to increased larval development time in *H. melpomene* in lowland individuals (compared to highland) and increased tolerance in *H. erato* lowland individuals (with no effect in highland individuals). This GXE interaction in *H. erato* was also reflected in their gene expression, where this increase in heat tolerance appeared to be related to the significant up-regulation of genes (in particular heat shock proteins)

with enriched GO terms associated with heat response and protein re/folding. This up-regulatory induction of heat shock protein appears to play a substantial role in heat tolerance in both *H. sara* and *H. erato.* However, the overall effect of temperature on baseline gene expression is broad and complex, requiring further investigations to untangle the mechanisms that underlie acclimation. Through this comprehensive investigation linking phenotype to genotype, this thesis has laid the foundation for understanding heat response and adaptation across insect species in the neotropics, providing us with insight into possible climate change scenarios and implications.

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