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

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Conflict of interest

We have no conflict of interest to declare.

Author contributions

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Statement on Inclusion

Our study brings together authors from several different countries, including scientists based in the country where the study was carried out. All authors were engaged early on with the research and study design to ensure that the diverse sets of perspectives they represent was considered from the onset.

Data availability

The data and scripts used for analyses are available on Zenodo,

doi:[10.5281/zenodo.16754980](https://doi.org/10.5281/zenodo.16754980)

Full details of all the statistical models and effect sizes are included in the Supplementary Tables Excel file (also included as separate .csv files).

All wing photographs are available on Zenodo, under the following DOIs: *Heliconius erato*

dorsal wing images from common garden experiment, doi:[10.5281/zenodo.16688551](https://doi.org/10.5281/zenodo.16688551),

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transplant experiment: doi:[10.5281/zenodo.16762443](https://doi.org/10.5281/zenodo.16762443), doi:[10.5281/zenodo.16762500](https://doi.org/10.5281/zenodo.16762500);

Heliconius melpomene dorsal wing images from reciprocal transplant experiment,

doi:[10.5281/zenodo.16762538](https://doi.org/10.5281/zenodo.16762538), doi:[10.5281/zenodo.16762573](https://doi.org/10.5281/zenodo.16762573)

Abstract

Climatic stratifications, in particular differences in temperature, occur along altitudinal clines.

Understanding divergence across these regions can give insight into speciation and diversification, as well as how species may respond to climate change scenarios. Most past research has focused on temperate regions, yet 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 investigate how genetics and the environment influence a wide range of traits in two widespread neotropical butterfly species, *Heliconius erato* and *Heliconius melpomene*, which occur across an altitudinal cline in the Andes. Using 'common garden' rearing of over 1,000 offspring from over 70 wild females caught along an altitudinal gradient, as well as rearing of populations from either end of the altitudinal range in their reciprocal temperature environments, we investigate genetic, environmental and gene-by-environment interaction effects on developmental, morphological, and thermal tolerance traits. We find parallel divergence in wing colour in both species, with darker colours at higher altitude, consistent with a role in thermoregulation. We also find evidence for gene-by-environment interactions: In *H. erato* we found local differences in heat acclimation response, with increased heat knock-out times at higher rearing temperatures found only in low altitude populations, which are exposed to the hottest temperatures. We find evidence for heritable genetic variation in most traits measured, with positive implications for adaptation to climate change, although our results suggest that selection may not act in a straightforward way on these traits.

Key words: Altitude, animal colour, butterfly, elevation, local adaptation, phenotypic plasticity, thermal biology, tropical ecology

Introduction

Ever since Humboldt drew the attention of the scientific world to the recurrent morphologies that plants show along altitudinal gradients (Humboldt & Bonpland, 1807), 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). Seminal work by Janzen (Janzen, 1967) predicted the effect of altitude on speciation and diversification to be more important in the tropics, but this has rarely been thoroughly tested (Ghalambor, 2006). In the tropics, climate not only varies rapidly with altitude but, due to the lack of seasonality, it varies little throughout the year. This creates 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, altitudinal adaptation in the tropics has still attracted relatively little attention (Slade & 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 show increased body size with altitude (Sullivan & Miller, 2007) consistent with “Bergmann’s rule” (Bergmann, 1848), which states that animals tend to have larger body sizes in colder environments, perhaps because larger body sizes increase cold tolerance (Scharf et al., 2014). Wing morphologies can also show variation with altitude. Wing loading has been found to increase across tropical moth taxa Geometridae and Arctiinae as well as in the temperate butterfly species *Coenonympha* in association with alternative flight

strategies possibly due to increasing wind speeds at higher altitudes (Brehm et al., 2019; Nève & Després, 2020). Wing colouration has been shown to darken at higher altitude in *Catasticta* butterfly species which was associated with increased heating rates (Dufour et al., 2018). This is consistent with a more general trend found in a range of ectotherms of darker individuals being found in colder environments, known as the thermal melanism hypothesis or Bogert's Rule (Bogert, 1949; Clusella Trullas et al., 2007).

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 temperate butterfly species, for example *Lycaena tityrus*, when reared in 'common garden' (standardised) conditions, show differences in development time, heat-stress resistance, and flight duration between populations (Karl et al., 2008), while *Colias eriphyle* exhibits gene-by-environment interactions in wing solar absorptivity across an altitudinal gradient (Kingsolver & 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 has been observed in butterflies such as *Melitaea cinxia* with

regard to metabolic rates (Niitepõld, 2010) and in *Bicyclus anynana* with developmental growth rates (Brakefield & Kesbeke, 1997).

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 in learning how living organisms may cope with long-term climatic changes (such as global warming) that are substantially altering the climate around the globe (Gienapp et al., 2008). This is because while phenotypic plasticity allows for rapid adjustment to new or fluctuating environmental conditions providing immediate benefits to enhance survival, it is typically non-heritable and can be costly to maintain long term (Murren et al., 2015). For example, a common plastic response to temperature stress is acclimation, where previous minor exposure to temperature stress leads to a priming response (Leroi et al., 1994; Wilson & Franklin, 2002). This typically induces heat shock proteins (HSPs) more rapidly, often at a higher concentration, during future stress, thus allowing for a greater stress tolerance threshold (Sejerkilde et al., 2003). However, HSP production is energetically costly, and constant maintenance can be detrimental to survival, as observed in *B. anynana*, where long-term exposure to heat has been shown to impair later heat tolerance (Fischer et al., 2010). Genetic variation in plasticity can be selected for and can be observed in populations across altitudinal gradients. For example, *L. tityrus* shows altitudinal differences in *hsp70* regulation in relation to rearing temperature, with lowland populations reared at higher temperatures significantly up-regulating *hsp70* levels (Franke et al., 2019). Thus, even though the production of HSPs is energetically costly, there is a clear selection to maintain high HSP levels in warmer lowland environments rather than solely during acute stress. This highlights the importance of genetic plasticity in enabling long-term adaptation to changing environments.

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 on the Eastern slopes of the Andes in Ecuador (Figure 1). The co-mimicking species *H. erato* and *H. melpomene* have a largely overlapping and wide distribution across the neotropics spanning an altitudinal range from sea level to just over 1000 meters above sea level (m.a.s.l.) (Rosser et al., 2012). While this altitudinal range is not particularly great, it is sufficient for populations at either end of the range to experience largely non-overlapping temperature variation, with the higher altitude sites being on average 4.1°C cooler (Montejo-Kovacevich et al., 2020). This makes it an excellent test for the prediction that adaptation may occur over narrow altitudinal ranges in the tropics. In addition, these two species are distantly related and non-interbreeding, making them a useful study system for investigating repeated adaptation, where parallel divergence in both species can more confidently be attributed to adaptation, rather than random variation or drift (Montejo-Kovacevich et al., 2022). 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., 2021; Montejo-Kovacevich et al., 2019), as well as evidence for adaptive genetic differences with altitude, some of which have been acquired via introgression from other species (Montejo-Kovacevich et al., 2022). Previous work also found some evidence for variation in heat tolerance, although this appeared to be primarily due to plasticity (Montejo-Kovacevich et al., 2020), warranting further investigation.

Through controlled temperature rearing experiments, we aimed to disentangle the roles of genetics and plasticity in these species with regard to a range of morphological,

developmental and thermal tolerance traits. The presence of genetically-controlled phenotypic differences between populations would be evidence of local adaptation and support the hypothesis that species in the tropics can and do adapt to relatively narrow altitudinal clines. In contrast, tropical species have been suggested to be less likely to show adaptive plasticity in response to temperature, due to the presence of stable year-round temperatures, meaning there should be less adaptive benefit of altering the phenotype in response to temperature (Ghalambor, 2006).

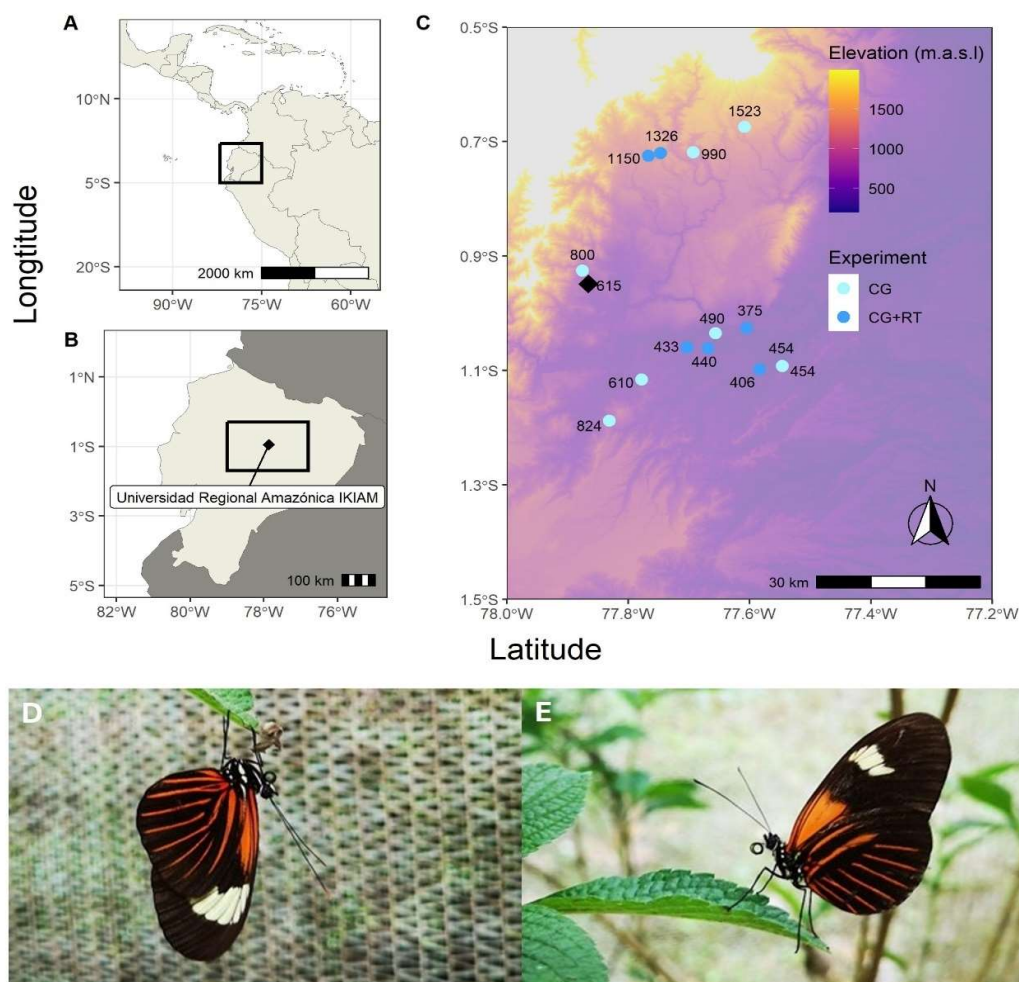


Figure 1: Capture sites and rearing location of butterflies sampled for the 'Common Garden' (CG) and 'Reciprocal Transplant' (RT) experiments. Location of the study within South America

(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. Photographs of the study species, *H. erato lativitta* (D) and *H. melpomene malleti* (E) in the study facility at Ikiam.

ALT TEXT: Map of study location showing sampling points. Images of the two study species.

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. The first experiment, a Common Garden Experiment (CGE), involved individually rearing the offspring of wild-caught females 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 took place under controlled laboratory conditions at the Amazonian Regional University Ikiam (Figure 1B). Laboratory temperature was set to be approximately intermediate between the temperatures observed at either end of the altitudinal range (Montejo-Kovacevich et al., 2020): 21.2 ± 1.1 °C (mean $\pm$ sd). Humidity was monitored but not strictly controlled, and the light regime followed the natural day-night cycles at this latitude (~12:12 hours). This allowed us to assess the heritable differences associated with altitude, controlling for plasticity.

The second experiment, a reciprocal transplant experiment (RTE), involved rearing offspring from wild-caught females from two altitude extremes, approximately $400 \pm 50\text{m}$ and $1200 \pm 100\text{m}$ above sea level (Figure 1C). For convenience, we will hereafter refer to butterflies reared from individuals captured below 450 m.a.s.l. as “low altitude” and those reared from individuals captured above 1000 m.a.s.l. as “high altitude”, acknowledging that this is not particularly high in the context of the Andes. Offspring from both altitudes were reared in growth chambers under two temperature treatments that replicated the high altitude and low altitude forest understory conditions (Montejo-Kovacevich et al., 2020): $19.1 \pm 1.7^\circ\text{C}$ for $\sim 1200\text{m}$ and $23.5 \pm 2.1^\circ\text{C}$ for $\sim 400\text{m}$. A 12:12 hours day-night regime was implemented in both growth chambers. Chamber temperature conditions closely replicated natural conditions (Figure S1). This was, therefore, a fully-crossed design between rearing temperature and altitude and 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 heritable population differences in the extent or direction of plasticity), when individuals from different altitudes showed distinct responses in the two alternative temperature treatments.

In both experiments, collection sites for the mothers were restricted to the geographical range of the co-mimetic subspecies *H. erato lativitta* and *H. melpomene malleti*, to avoid potential confounding effects associated with subspecies colour pattern variation. Mothers were kept in shaded outdoor insectaries at Ikiam (600m above sea level). Eggs were collected daily in the late afternoon at the same time, moved to the lab, and separated into individual rearing containers. 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.

All work was conducted with permission of the Ecuadorian Government under the following permit numbers: MAE-DNB-CM-2018-0088 and MAATE-DBI-CM-2021-0176

Developmental parameters

Eggs, larvae, and pupae were 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 conducted 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 CGE adults, cold tolerance tests were conducted on the second day after eclosion 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 (as these individuals were used for a subsequent gene expression study).

Cold tolerance was tested by exposing the butterflies to 5 ± 1 °C in a refrigerator with a glass door, 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. All butterflies were chilled for 10 minutes regardless of when KO occurred to prevent raising the temperature of the refrigerator by opening the door. Ten minutes was selected based on pilot experiments and all individuals entered chill coma within this time. The knockout response involved the butterfly going from a vertical resting position with its wings upright to lying flat with its wings together on its side, which was generally quite consistent and obvious. Recovery occurred in the lab at 21°C and was characterised by the ability of the butterfly 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 Montejó-Kovacevich et al. (Montejó-Kovacevich et al., 2020), with minor modification. Knockout was defined as the point at which the butterfly could no longer stand up on at least 3 legs and the thorax was touching the floor of the chamber. Butterflies were removed from the heat immediately upon KO, and in the CGE recovery at 21°C was timed. After the heat tolerance experiment, all butterflies were killed and preserved. Butterflies that did not knockout during the experimental duration (120 minutes) were excluded from the analysis. To ensure that this did not bias the results we also conducted an analysis where these individuals were included in the analysis with KO time set to 128 minutes.

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 background removal. Wing size, and shape were measured from preserved wings analysed as in the methods in Montejo-Kovacevich et al. (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^2 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 (Figure S2). We specified that the images contained three distinct colours, as both species have distinct patches of black, red and yellow. ColourDistance clusters all pixels most similar to one another into the defined number of groups and calculates the proportion and mean R.G.B. values for each group (Weller & Westneat, 2019). Brightness of the red region (hereafter red brightness) was calculated by combining mean red, green and blue values in the red region and dividing by three to get an estimate of percentage reflectance (within the range of the camera's sensitivity). Red reflectance was not measured in the RTE due to mechanical damage in the red patch.

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 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), and '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 heritability 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 & 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 & 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 (due to the larger sample size), 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 (Nakagawa & Schielzeth, 2010), using package *rptR* (v.0.9.22)(Stoffel et al., 2017). The ICC is a measure of how much of the variation in the model is explained by the sibling relationships within each brood, due to genetic relatedness and maternal effects. An upper estimate of heritability is 2 * ICC for full siblings (Falconer & Mackay, 1996), assuming negligible maternal effects. Remating is rare in the wild in the two species studied (Walters et al., 2012), so it is most likely that individuals from the same mother are full siblings. ICC values were also calculated using the null models without any additional explanatory variables, to determine whether controlling for these influenced the heritability estimates.

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 et al., 2023).

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 "rnatrualearth" (Massicotte & South, 2023), "raster" (Hijmans, 2023), "sf" (Pebesma, 2018; Pebesma & Bivand, 2023) and "ggrepel" (Slowikowski, 2023).

Results

Differences between altitudinal populations

In the common garden experiment (CGE) for *H. erato*, there were between 29 and 45 families included in the analyses, depending on the trait examined (Table 1). Specifically, we analysed >400 individuals for development time and body size, 1549 individuals for larval survival, 802 for pupal survival, and >300 for wing morphology, colour, and thermal tolerance.

For *H. melpomene*, we analysed between 20 and 25 families across traits, with >400 individuals for development time and body size, >800 for survival, and >300 for other traits.

Table 1: The effect of altitude in the GCE, per species, per trait. Delta AICc and AICc weights are given for the best supported model that included altitude as fixed effect, relative to the best supported model (Table S1). Point estimates of the effect of altitude on each trait are given in standard deviation units with their 95% confidence intervals (plotted in Figure S4). The effect of altitude is considered significant (shown in bold) if it is retained in the best supported model (the model with the lowest AICc, or that with the fewest explanatory variables within 2 AICc units of the model with the lowest AICc) and the confidence interval estimates do not cross zero.

Species	Trait	No. mothers (broods)	No. ind.	$\Delta AICc$	wt AICc	Effect size	CIs
<i>H. erato</i>	Laval dev. time	38	775	1.35	0.34	-0.027	-0.093, 0.038
<i>H. melpomene</i>	Laval dev. time	25	450	1.54	0.32	-0.005	-0.055, 0.043
<i>H. erato</i>	pupal dev. time	38	558	1.74	0.3	0.016	-0.054, 0.087
<i>H. melpomene</i>	pupal dev. time	22	405	1.71	0.3	0.017	-0.045, 0.078
<i>H. erato</i>	pupa weight	40	753	1.58	0.21	-0.021	-0.084, 0.042
<i>H. melpomene</i>	pupa weight	23	444	2.02	0.24	-0.003	-0.046, 0.040
<i>H. erato</i>	adult weight	45	327	1.97	0.21	-0.013	-0.106, 0.080
<i>H. melpomene</i>	adult weight	29	293	1.79	0.12	-0.017	-0.083, 0.049
<i>H. erato</i>	Larval survival	42	1549	2	0.27	-0.004	-0.170, 0.157
<i>H. melpomene</i>	Larval survival	26	855	1.74	0.3	-0.026	-0.129, 0.079
<i>H. erato</i>	Pupal survival	40	802	2.01	0.27	-7.97E-4	-0.064, 0.051
<i>H. melpomene</i>	Pupal survival	24	495	0.77	0.4	-0.041	-0.124, 0.035
<i>H. erato</i>	wing load	44	319	1.77	0.19	-0.026	-0.126, 0.074
<i>H. melpomene</i>	wing load	29	290	1.48	0.19	-0.031	-0.115, 0.052
<i>H. erato</i>	black %	36	315	0.5	0.14	0.001	4.70E-4, 0.002
<i>H. melpomene</i>	black %	23	333	1.63	0.1	6.09E-4	6.62E-5, 0.001
<i>H. erato</i>	red reflectance	33	416	0	0.44	-3.22E-4	-9.04E-4, 2.53E-4
<i>H. melpomene</i>	red reflectance	22	431	0	0.75	4.27E-4	-3.07E-7, 8.45E-4
<i>H. erato</i>	Cold KO	37	436	0	0.14	0.009	-0.032, 0.049
<i>H. melpomene</i>	Cold KO	23	374	0	0.07	-0.017	-0.055, 0.021
<i>H. erato</i>	Cold Recovery	37	436	0	0.6	0.031	-0.026, 0.088
<i>H. melpomene</i>	Cold Recovery	23	374	0	0.6	-0.014	-0.047, 0.018
<i>H. erato</i>	Heat KO	31	297	0	0.15	2.033	0.303, 3.665
<i>H. melpomene</i>	Heat KO	20	239	0.37	0.07	0.014	-0.084, 0.112
<i>H. erato</i>	Heat Recovery	31	297	0	0.09	-0.357	-0.667, -0.046
<i>H. melpomene</i>	Heat Recovery	20	239	0.81	0.04	-0.007	-0.044, 0.031

The traits that showed statistically supported differences between populations across altitudes were wing colouration and heat tolerance (Table 1). In both *H. erato* and *H. melpomene* the percentage of the forewing that is black increased with altitude (Figure 2), with the best supported models for both species including altitude as an explanatory variable (*H. erato* AICcWt = 0.14; *H. melpomene* AICcWt = 0.1, Table S1). In *H. erato* the percentage of the wing that was black was on average 2% more in the offspring of mothers collected at or above 1000 m.a.s.l. relative to those collected at 400 m.a.s.l., while for *H. melpomene* 1.6% more of the wing surface was black (Figure 2). This was due to a reduction in the relative size of both the red and yellow wing patches (Figure S3). The increase in black percentage is consistent with the thermal melanism hypothesis and findings in other species (Clusella Trullas et al., 2007; Dufour et al., 2018) that higher altitude populations are often darker, possibly to improve heating ability in cooler environments.

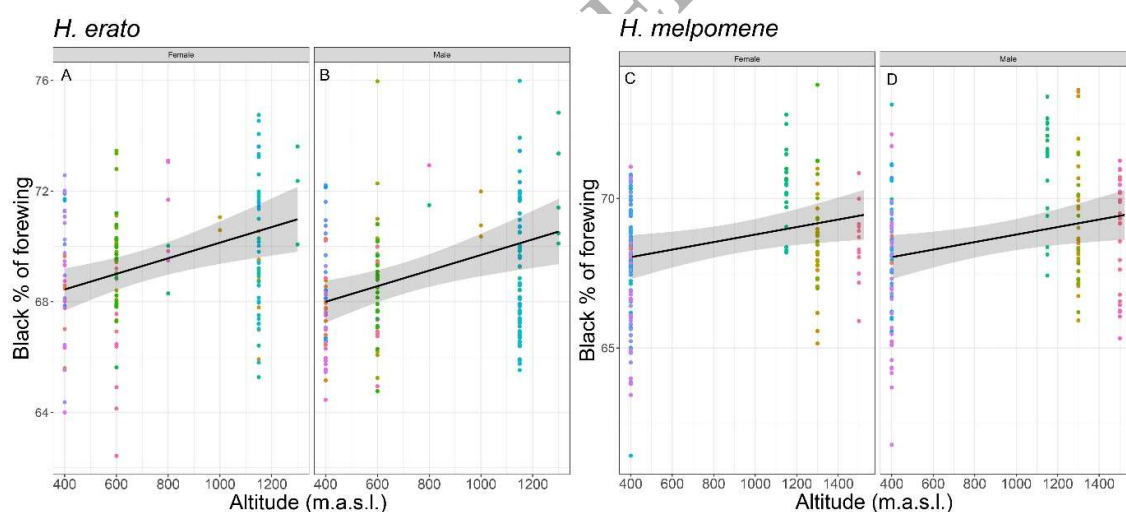


Figure 2: The relationship between black percentage of the wing and altitude of the source population in *H. erato* females (A) and males (B) and *H. melpomene* females (C) and males

(D). Points are individuals coloured by mother ID (points of the same colour are siblings).

Model fit lines and 95% confidence intervals are shown. For *H. melpomene*, sex was not included in the best-supported model, so the lines plotted in C and D are the same.

ALT TEXT: Graphs showing a positive relationship between altitude and black % of the forewing in both sexes of *H. erato* and *H. melpomene*.

Reflectance of the red wing patch showed no substantial difference with altitude in *H. erato*, (Table 1), with the highest ranked model containing only sex as a relevant explanatory factor (AICcWt = 0.34). However, in *H. melpomene*, the highest ranked model included altitude, but only when interacting with sex (AICcWt = 0.75, Table 1, Figure 3). Females from mothers collected at or above 1000 m.a.s.l. on average reflected 0.6% more visible light compared to those from mothers collected at 400 m.a.s.l., while males remained relatively unchanged across altitudes (Figure 3).

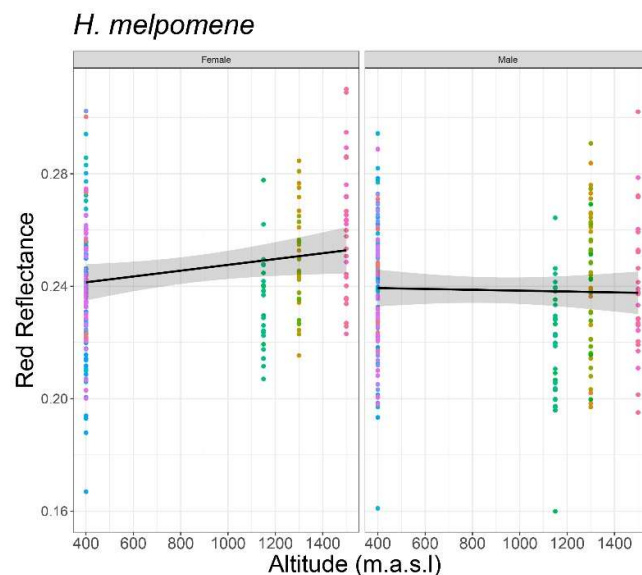


Figure 3: The relationship between reflectance of the forewing red wing patch (summed RGB values from colour photographs as a proportion of white values, where white = 1) with

altitude in *H. melpomene*, separated by sex. Points are individuals coloured by mother ID (points of the same colour are siblings). Model fit lines and 95% confidence intervals are shown.

ALT TEXT: Graphs showing a positive relationship between altitude and red reflectance in *H. melpomene* females, but no trend in males.

For heat tolerance, *H. erato* showed differences between populations across altitudes, where individuals from higher altitude took longer to enter heat coma (hereafter referred to as heat knock-out or KO), and less time to recover from KO when removed from the heat. Both patterns suggest an increased heat tolerance at higher altitudes, contrary to our expectations (Figure 4, Figure S4). Individuals from mothers sampled above 1000 m.a.s.l. took on average 1.4 more minutes to knock out and 4.4 fewer minutes to recover, compared to individuals from mothers sampled at 400 m.a.s.l. (Figure 4). While this difference does appear to be quite small given the range of the data, it was statistically supported once accounting for black percentage and body weight. The highest ranked models for both traits included altitude as an explanatory factor (heat KO: AICcWT = 0.14; heat recovery AICcWT = 0.09, Table S1). For heat KO, the best supported model also contained black percentage, body weight, and their interactions with altitude. A higher black percentage increased the time to KO (effect size = $229.58x \pm 204.81$) while decreasing recovery time (effect size = $-11.41x \pm 7.41$), both suggesting increased heat tolerance, again contrary to expectations under the thermal melanism hypothesis. Increased body size reduced heat recovery time (effect size = $-7.70x \pm 5.28$), suggesting larger individuals may be more heat tolerant. However, body size also had a negative, but non-significant effect on KO time (effect size = $-2.02x \pm 2.08$).

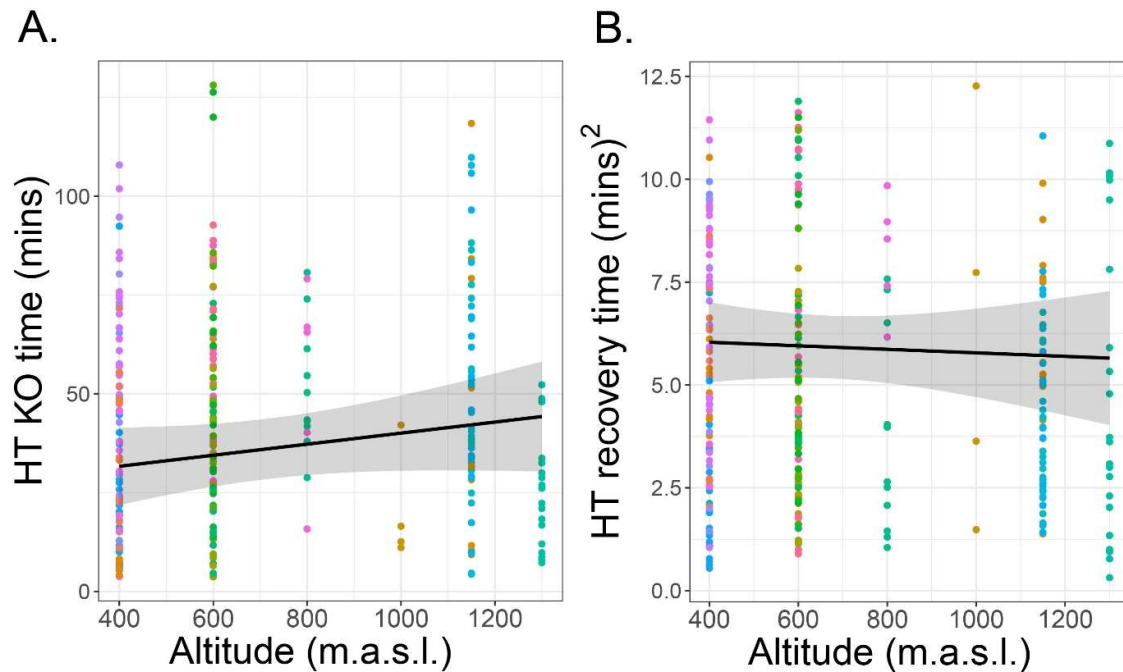


Figure 4: The relationships between heat knock-out time (A) and recovery time from knock-out (B) with altitude in *H. erato*. Longer knock-out times and shorter recovery times are both indicative of greater heat tolerance. Points are individuals coloured by mother ID (points of the same colour are siblings). Model fit lines and confidence intervals are shown.

ALT TEXT: Graphs showing a positive relationship between altitude and heat knock-out time (in panel A) and a negative relationship between altitude and recovery time from knock-out (in panel B) in *H. erato*.

In *H. melpomene*, the best supported models included an effect of altitude on heat KO and recovery (Table S1), but the effect sizes were small, and the confidence intervals crossed zero (Figure 2D), suggesting the effect is not statistically significant. The models also supported an effect of body size: larger individuals exhibited a shorter time to KO (effect size = $-6.73x \pm 4.11$), suggesting reduced heat tolerance. However, body size had no detectable effect on recovery time (effect size = $0.69x \pm 2.40$).

To ensure that excluding individuals that did not knock out did not bias our results, we repeated our analysis including these individuals with KO time set to 128 minutes, which added 70 individuals for *H. erato* and 36 for *H. melpomene*. The best supported models were largely the same (still contained altitude as an explanatory factor, Table S2) and the effect of altitude in *H. erato* was similar (effect size = $2.41x \pm 1.55$) and was still negligible in *H. melpomene* (effect size = $0.01x \pm 0.05$).

For cold tolerance, the models for both species also supported an effect of altitude on time to enter chill-coma (cold KO), and for *H. melpomene* on the time to recover from chill-coma (Table S1). However, the effect size estimates were small and crossed zero in all cases (Table 1). The models also supported an effect of body size on cold KO in both species, with larger individuals taking longer to KO, suggesting they are more cold-tolerant (*H. erato*: effect size = $3.11x \pm 1.54$; *H. melpomene*: effect size = $2.25x \pm 1.82$). This was also supported in *H. melpomene* cold recovery time, where larger individuals recovered faster (effect size = $-4.72x \pm 1.83$). The effects of all modelled fixed effects on each trait are given the Supplementary Tables.

All altitudinally-associated traits had high and significant intraclass correlation coefficients (ICC) in the CGE broods (Table 2). All individuals were reared individually under identical conditions, so similarity within broods can only be attributed to shared genetic variation and maternal effects. If this is primarily due to genetic effects, heritability can be approximated as two times the ICC (Falconer & Mackay, 1996), bearing in mind that this will likely be inflated by any maternal effects, which we could not distinguish in our experimental design. Based on

these estimates, black wing percentage has a very high heritability (0.99 in *H. erato* and 0.71 in *H. melpomene*) and heat tolerance in *H. erato* has fairly high heritability (0.61 and 0.51 for heat knock-out and recovery respectively) (Table 2). This suggests that our CGE was detecting genetic differences between populations, consistent with local adaptation to altitude. Although maternal effects likely mean that these heritability estimates are inflated (Moore et al., 2019), any half-sibs present (which based on knowledge of *Heliconius* mating systems is unlikely but possible (Walters et al., 2012)) would cause an underestimation of heritability. ICC values from null models without any fixed effects were very similar (Table S3), and in most cases were higher, suggesting that controlling for correlated factors in the model generally produced more conservative heritability estimates.

Even for traits that did not differ between populations at different altitudes, most had significant ICC values (Table 2). Of the traits we measured, the only ones that did not show significant ICC were cold KO time in *H. erato*, heat and cold recovery in *H. melpomene*, and pupal survival in either species (Table 2). There were differences in larval survival of offspring coming from different mothers (*H. erato*: ICC = 0.345, $p = 3.15E-72$, *H. melpomene*: ICC = 0.202 $p = 2.23E-13$, Table 2), but there was not a significant difference with altitude (Table 1), demonstrating that any phenotypic differences we observed with altitude were not due to selective filtering of genotypes during the experiment.

Table 2: Intraclass correlation coefficients (ICC, R) and their 95% confidence intervals (CIs), and p-values, for each trait in each species (those in bold also differed with altitude). These were used to give an upper estimate of heritability as $2 \times \text{ICC}$

<i>Species</i>	<i>Trait</i>	<i>ICC (R)</i>	<i>CIs</i>	<i>p value</i>	<i>h2 (upper estimate)</i>
<i>H. erato</i>	Laval dev. time	0.365	0.245,0.475	1.10E-44	0.73
<i>H. melpomene</i>	Laval dev. time	0.217	0.085,0.337	9.01E-12	0.43
<i>H. erato</i>	pupal dev. time	0.348	0.211,0.467	1.13E-31	0.70
<i>H. melpomene</i>	pupal dev. time	0.379	0.212,0.537	2.84E-23	0.76
<i>H. erato</i>	pupa weight	0.329	0.213,0.438	1.24E-40	0.66
<i>H. melpomene</i>	pupa weight	0.182	0.067,0.303	5.54E-10	0.36
<i>H. erato</i>	adult weight	0.431	0.249,0.577	6.60E-22	0.86
<i>H. melpomene</i>	adult weight	0.183	0.046,0.333	3.25E-06	0.37
<i>H. erato</i>	Larval survival	0.369	0.369, 0.369	2.43E-73	0.74
<i>H. melpomene</i>	Larval survival	0.207	0.207, 0.207	8.28E-14	0.41
<i>H. erato</i>	Pupal survival	0.003	0.003, 0.003	0.395	0.01
<i>H. melpomene</i>	Pupal survival	0.010	0.01, 0.01	0.292	0.02
<i>H. erato</i>	wing load	0.437	0.253,0.592	5.68E-25	0.87
<i>H. melpomene</i>	wing load	0.392	0.16,0.567	5.28E-11	0.78
<i>H. erato</i>	black %	0.495	0.342,0.624	1.26E-32	0.99
<i>H. melpomene</i>	black %	0.355	0.185,0.506	3.57E-22	0.71
<i>H. erato</i>	red reflectance	0.254	0.127,0.372	1.67E-14	0.51
<i>H. melpomene</i>	red reflectance	0.132	0.038,0.244	6.58E-08	0.26
<i>H. erato</i>	Cold KO	0.033	0,0.114	0.195	0.07
<i>H. melpomene</i>	Cold KO	0.105	0.01,0.219	0.002	0.21
<i>H. erato</i>	Cold Recovery	0.118	0.014,0.225	5.35E-05	0.24
<i>H. melpomene</i>	Cold Recovery	0.054	0,0.14	0.091	0.11
<i>H. erato</i>	Heat KO	0.307	0.112,0.472	3.90E-05	0.61
<i>H. melpomene</i>	Heat KO	0.250	0.066,0.417	1.40E-05	0.50
<i>H. erato</i>	Heat Recovery	0.256	0.082,0.422	1.67E-03	0.51
<i>H. melpomene</i>	Heat Recovery	0.043	0,0.162	0.196	0.09

Temperature effects on traits and differences between populations

In the reciprocal transplant experiment (RTE) there were >14 families analysed in *H. erato* with >200 individuals analysed for survival and >100 for all other traits between both altitudes. In *H. melpomene*, there were >6 families analysed with >100 individuals for survival, >30 individuals for thermal tolerance and >50 individuals for all other traits (Table 3). The much smaller sample size in *H. melpomene*, especially in lowland broods (Table S4), meant we had less power to discern population differences or their interaction with

temperature compared to *H. erato*. See Table S4 for full details of models, and the sample sizes included for each trait.

Table 3: The effect of rearing temperature in the RTE, per population, per species, per trait. Delta AICc and AICc weights are given for the best supported model that included both temperature and altitude as fixed effects, relative to the best supported model (Table S4). The effect of temperature (as a ratio of the trait value at 24°C/19°C) is given with the 95% confidence intervals (plotted in Figure S5). Significant effects of temperature are shown in bold, and significant interactions between temperature and altitude are indicated with a star. Significance was determined as effects retained in the best supported model (the model with the lowest AICc, or that with the fewest explanatory variables within 2 AICc units of the model with the lowest AICc) and with confidence interval estimates that did not cross one.

Species	Trait	No. mothers (broods)	No. ind.	$\Delta AICc$	wt AICc	Pop alt.	Temp Effect Size	CIs
H. erato	Larval dev. time	16	199	1.92	0.2	400m	0.600	0.571, 0.63
						1200m	0.610	0.58, 0.641
H. melp.	Larval dev. time*	8	79	0	0.78	400m	0.588	0.545, 0.631
						1200m	0.679	0.631, 0.726
H. erato	Pupal dev. time	15	143	0	0.35	400m	0.505	0.485, 0.525
						1200m	0.535	0.515, 0.556
H. melp.	Pupal dev. time	8	68	3.99	0.06	400m	0.510	0.484, 0.536
						1200m	0.523	0.498, 0.547
H. erato	Pupa weight	16	196	1.82	0.18	400m	0.904	0.767, 1.04
						1200m	0.835	0.709, 0.96
H. melp.	Pupa weight	8	75	1.34	0.29	400m	0.796	0.67, 0.922
						1200m	0.887	0.785, 0.989
H. erato	Adult weight	15	131	3.41	0.05	400m	0.823	0.733, 0.913
						1200m	0.874	0.767, 0.981
H. melp.	Adult weight	8	58	0	0.27	400m	0.675	0.449, 0.901
						1200m	0.905	0.697, 1.113
H. erato	Larval Survival	16	297	4.25	0.06	400m	0.919	0.738, 1.101
						1200m	1.024	0.892, 1.156
H. melp.	Larval Survival	9	155	3.2	0.08	400m	1.305	0.476, 2.133
						1200m	1.099	0.848, 1.349
H. erato	Pupal Survival	16	200	5.66	0.03	400m	1.114	0.78, 1.448
						1200m	1.008	0.716, 1.301
H. melp.	Pupal Survival	9	116	3.72	0.06	400m	0.890	0.659, 1.121
						1200m	0.921	0.737, 1.106
H. erato	Wing Load	14	127	4.68	0.02	400m	1.084	0.948, 1.221
						1200m	1.053	0.928, 1.177
H. melp.	Wing Load	8	87	1.58	0.06	400m	1.182	0.958, 1.406
						1200m	1.188	0.956, 1.421
H. erato	Aspect ratio	14	127	6.06	0.01	400m	1.005	0.987, 1.022
						1200m	1.005	0.989, 1.022
H. melp.	Aspect ratio	8	87	9.61	0	400m	0.989	0.975, 1.002
						1200m	0.988	0.975, 1.002
H. erato	Black %	13	92	0	0.19	400m	0.986	0.965, 1.007
						1200m	1.016	0.998, 1.035
H. melp.	Black %	8	75	0	0.14	400m	1.009	0.995, 1.023
						1200m	1.009	0.995, 1.023
H. erato	Cold KO	12	35	6.23	0.02	400m	0.816	0.605, 1.027
						1200m	0.703	0.482, 0.923
H. melp.	Cold KO	6	23	4.19	0.03	400m	0.796	0.427, 1.165
						1200m	1.378	0.838, 1.918
H. erato	Cold recovery	12	35	3.95	0.07	400m	1.065	0.502, 1.629
						1200m	0.758	0.338, 1.179
H. melp.	Cold recovery	6	23	6.12	0.02	400m	2.377	0.41, 4.345
						1200m	1.082	0.376, 1.789
H. erato	Heat KO*	12	45	0	0.67	400m	2.127	1.332, 2.922

H. melp.	Heat KO	6	35	0	0.35	1200m	1.116	0.742, 1.49
						400m	1.123	0.499, 1.748
						1200m	1.578	0.854, 2.303

As expected, rearing temperature had a significant effect on development time and body size in both species (Table 3). In *H. erato* and *H. melpomene* larval and pupal development time both roughly halved at the higher rearing temperature of 24°C compared to at 19°C (effect sizes between 0.51 and 0.69, Table 3). Some marginal population differences were observed in *H. erato*, but these were within 2 AICc units of the models with temperature alone (Table S4), and also went in opposite directions in larvae and pupae, with low altitude larvae developing slightly faster under both temperatures (something that was not observed in the CGE), and high altitude pupae developing faster, but only at 19°C (Figure S6). In *H. melpomene* there was no effect of altitude on pupal development time and the model with temperature alone was the highest ranked (AICcWt = 0.46) (Table 3, Table S4). However, for larval development time, the highest ranked model supported an interaction between altitude and temperature (AICcWt = 0.78), with lowland populations showing greater differences in their developmental time with temperature (24°C:19°C, effect size = $0.59x \pm 0.04$) as compared to highland populations (24°C:19°C, effect size = $0.69x \pm 0.05$) (Table 3, Figure 5). This is consistent with an adaptive difference in *H. melpomene*, whereby high-altitude populations are able to develop faster at colder temperatures.

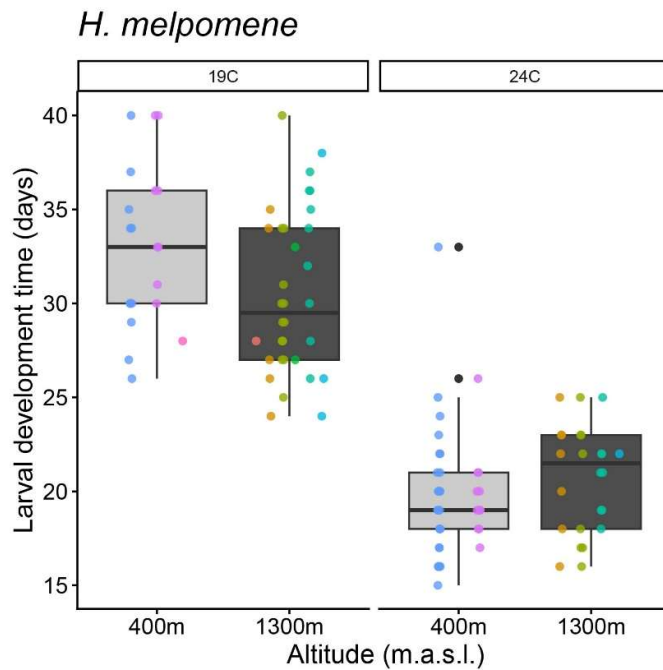


Figure 5: The effect of rearing temperature (top bar) and source population altitude on larval development time in *H. melpomene*. Boxes show the median and interquartile ranges for each population under each treatment, with overlain points for each individual. Boxes are coloured by population (light grey 400 m.a.s.l., dark grey 1300 m.a.s.l.); points are coloured by mother ID (i.e. points of the same colour are siblings).

ALT TEXT: Graphs showing the effect of rearing temperature and altitude on larval development time in *H. melpomene*, illustrated with box plots and individual data points.

Temperature has the largest effect but it is greater in the 400m population than the 1300m population.

We measured the effect of temperature on body size by weighing pupae one day after pupation and by weighing adults one day after eclosion, before feeding. Across both species, higher rearing temperatures tended to result in smaller pupae and adults (Table 3). There were some apparent differences in the effect sizes between populations, but the best

supported model for *H. erato* pupal weight contained only temperature interacting with development time as explanatory factors (AICcWt = 0.46) and for adult weight contained temperature alone (AICcWt = 0.11, Δ AICc = 1.61). For *H. melpomene* pupa weight, the highest ranked model contained both temperature and altitude as explanatory variables (without an interaction, AICcWt = 0.56, Table S4). 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 = $0.80x \pm 0.13$, highland effect size = $0.887x \pm 0.102$) with no clear altitudinal effects (Figure S6). For adult weight in *H. melpomene*, our highest ranked model again contained an interaction between temperature and altitude (AICcWt = 0.27) but this interaction effect was marginal as this model was not stronger than the model without the interaction term (Delta_AICc = 0.48, AICcWt = 0.21). Therefore, the results for *H. melpomene* are similar to *H. erato*, with a lack of strong evidence for population differences in the effects of rearing temperature on body size.

We found evidence of cold acclimation *H. erato*, but not *H. melpomene* (Table 3), whereby individuals reared at cooler temperatures took longer to enter chill coma than those reared at warmer temperatures (Figure S5C). This acclimation was only observed in highland populations (24°C:19°C: $0.70x \pm 0.22$), and not the lowland populations (24°C:19°C: $0.81x \pm 0.21$) (Table 3). However, the interaction was not statistically supported as the highest ranked model had temperature as the sole explanatory factor in time to chill coma (AICcWT = 0.51). We also found evidence of heat acclimation in *H. erato*, but again not in *H. melpomene*, whereby individuals reared at warmer temperatures took longer to enter heat coma than those reared at lower temperatures (Table 3). This effect was only seen in the low

altitude populations ($24^{\circ}\text{C}:19^{\circ}\text{C}$: $2.13x \pm 0.79$) not those from higher altitude ($24^{\circ}\text{C}:19^{\circ}\text{C}$: $1.12x \pm 0.37$) (Figure 6), and this population difference was statistically supported, with the highest ranked model including the interaction between temperature and altitude ($\text{AICcWT} = 0.67$, Table S4). This is consistent with adaptive differences in plasticity between populations, with lower altitude populations being better adapted to respond to higher temperatures.

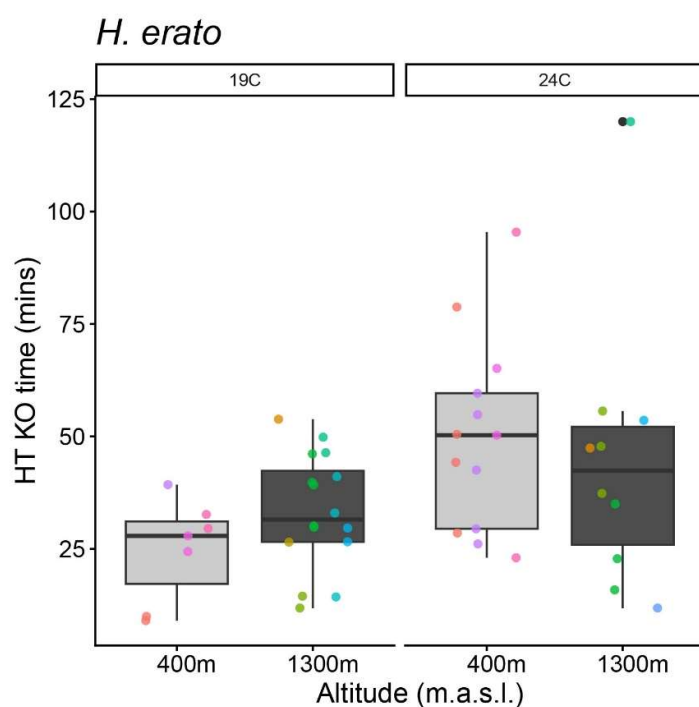


Figure 6: The effect of rearing temperature (top bar) and source population altitude on time to heat knock-out at 39°C in *H. erato*. Boxes show the median and interquartile ranges for each population under each treatment, with overlain points for each individual. Boxes are coloured by population (light grey 400 m.a.s.l., dark grey 1300 m.a.s.l.); points are coloured by mother ID (i.e. points of the same colour are siblings).

ALT TEXT: Graphs showing the effect of rearing temperature and altitude on heat knock-out (HT KO) time in *H. erato*, illustrated with box plots and individual data points. HT KO time is

greater when reared at 24°C in the 400m population but there is no difference in 1300m population.

In *H. erato* there was some evidence for an effect of rearing temperature on the percentage of the wing that was black, although the effect was small and confidence intervals for both populations crossed one (Table 3). However, the population difference that was found in the CGE was only found when individuals were reared at 24°C, being darker in the higher altitude population (400m:1300m = $0.95x \pm 0.03$) (Figure S6), with no difference in black wing percentage between populations when reared at 19°C (400m:1300m = $0.98x \pm 0.03$) (Figure S6). This was due to greater plasticity in the higher altitude population, with butterflies being darker when reared at higher temperatures, which was not found in the low altitude population (Figure S5). Our highest ranked model supported this interaction (AICcWt = 0.19) and also suggested an interaction effect between temperature and sex, where females had greater black percentage at higher rearing temperatures in comparison to males. Therefore, the population difference observed in the CGE is possibly complicated by differences in the extent of plasticity in wing colour in response to rearing temperature. In *H. melpomene* we did not detect any effect of rearing temperature on black percentage of the wing.

We again did not find any difference between populations in larval or pupal survival (Figure S6), demonstrating that selection operating during our experiment was not a factor in explaining between-population differences (or lack thereof). We also did not find any difference in survival between populations at the different rearing temperatures (Table 3), suggesting that the mean temperatures at the different altitudes are not causing differential selection between populations during the larval or pupal stages.

Discussion

Wing colour in *Heliconius* has been extensively studied, primarily in the context of Müllerian mimicry, and several genes that underlie pattern variation have been identified (Dasmahapatra et al., 2012; Nadeau, 2016). Here we have found evidence in both species of quantitative, highly heritable variation in the proportion of black pigmentation on the wing associated with altitude. While it is possible that this colour variation could be driven by differences in recognition and avoidance by predators across the altitudinal cline, as in other clines in *Heliconius* colour pattern variation (Mallet & Barton, 1989), the variation is quite subtle (1-2% overall difference) and well within the range of general inter-individual variation, making it quite unlikely that predators would perceive or discriminate based on this variation. On the other hand, in other butterfly species, fairly slight differences in melanisation have been shown to influence thermoregulation and fitness under different temperatures (Dufour et al., 2018; Rosa & Saastamoinen, 2020). It is also interesting that the increase in black comes from a decrease in both the red and yellow regions of the wing, which may also contraindicate a signalling function, where a change in the size or shape of a single colour patch might be more likely. The proximal location of the red patch is a region of the wing that in other species is important for thermoregulation (Ellers & Boggs, 2004); while the yellow wing patch on *H. erato lativitta* wings was found to be cooler under direct insolation, compared to the red and black wing regions, which were not significantly different (Ladwa et al., 2026). Therefore, reducing the relative sizes of both paler pigmented regions may increase heat gain when basking.

Similar trends of darker colours with increasing altitude or latitude have been found both within and between species in many insects, including butterflies (Günter et al., 2019) and are generally thought to be explained by thermal melanism, whereby darker individuals warm up faster when exposed to radiative heat (Clusella Trullas et al., 2007). In some species the differences are more pronounced than we find here (Günter et al., 2019; Roland, 1982), while in others the differences do appear to more be more subtle and not dissimilar to what we observe (Ellers & Boggs, 2002; González-Tokman et al., 2020; Guppy, 1986). Most of this data has come from temperate systems, where cold temperatures are likely to be more extreme and limiting, so it is interesting that we also observe this trend in tropical insects. A previous study of tropical butterflies in Ecuador found conflicting results, with one genus (*Catantix*) having darker species at higher altitudes, while another (*Leptoprobia*) showed the opposite trend (Dufour et al., 2018). We are not aware of any previous studies that have found a similar within-species trend in tropical butterflies.

While the colour variation we observe is consistent with thermal melanism, in *H. erato* this pigmentation had the opposite effect to expectations in the thermal tolerance experiments, where it appeared to increase heat tolerance. One explanation for this could be the artificial conditions of our experiment, where heating was purely via conduction and convection, while in nature, solar radiation is a major heat source (Barton & Porter, 2014), particularly in the context of thermal melanism, where basking behaviour is thought to be the main mode of action (Clusella Trullas et al., 2007). Nevertheless, in temperate systems, cold temperature is likely to be a strong selective agent at higher altitudes, but in tropical systems increasing solar radiation may be more important. Increasing solar radiation has been suggested to explain

why some species may show reversed trends of lighter colour with higher altitude, to prevent overheating under high solar radiation (Kuyucu et al., 2018).

In both species we find interesting sex differences in the reflectance of the red wing patch, consistent with previous work on these species showing subtle sexual dimorphism (Klein & de Araújo, 2013), which may function in mate choice. In *H. melpomene* we also find an interaction between sex and altitude, with *H. melpomene* females from higher altitude being more reflective. This could be consistent with females adapting to increased solar radiation at higher altitude, by reducing absorbance of heat or damaging wavelengths. Males may be more constrained by sexual selection, or less adapted to local conditions due to their higher dispersal (Jiggins, 2017).

Overall, we find heritable differences between populations in wing colour, building on previous work demonstrating heritable differences in wing shape between the same populations (Montejo-Kovacevich et al., 2021). High heritability of the wing colour variation we observed raises the interesting possibility that it may be controlled by some of the major colour pattern controlling genes that are known in these species (Nadeau, 2016), as has been found for other quantitative colour pattern variation (Bainbridge et al., 2020). While the differences in wing colour were clear in our common garden experiment, when reared under different temperatures in our reciprocal transplant experiment, we found that *H. erato* showed some evidence for thermally plastic differences in wing colour that differed with population and sex, which could make it harder to detect the colour difference in the wild, highlighting the benefits of disentangling genetic and environmental effects under controlled conditions.

A key assumption of Janzen's hypothesis (Janzen, 1967) that altitude has a greater impact in the tropics, is that tropical species will show narrow, altitudinally stratified thermal tolerance ranges (Ghalambor, 2006). We found limited support for this, with no difference in thermal tolerance between populations in *H. melpomene*. We did find significant differences between populations in heat tolerance in *H. erato*. However, in our common garden experiment these differences were in the opposite to expected direction (increased thermal tolerance in the high-altitude populations) and the opposite direction to that found previously by Montejo-Kovacevich et al. (Montejo-Kovacevich et al., 2020) in wild-caught individuals. This is likely due to the plasticity in this trait that we detected in our reciprocal transplant experiment, which also revealed differences in the extent of plasticity between populations. The low-altitude populations show an acclimation response, whereby rearing at higher temperatures causes them to be more heat tolerant as adults, but this response is not seen in the high-altitude populations. Therefore, when reared at 24°C, we find the expected population difference, with low-altitude populations being more heat tolerant. The opposite trend that we find in the common garden experiment is therefore likely due to low altitude populations becoming less heat tolerant when reared at the comparatively cool temperature of 21°C, while the high-altitude population remains relatively heat tolerant. This may also explain why Montejo-Kovacevich et al. (Montejo-Kovacevich et al., 2020) found no difference between populations in their common-garden experiment, as these were conducted outdoors at warmer and more variable temperatures.

Lack of a heat acclimation response in the high-altitude populations could be due to greater daily variation in temperatures seen in the high-altitude tropics, due to higher solar radiation

(Ghalambor, 2006), making it less beneficial for high-altitude populations to respond plastically to developmental temperature, while at lower altitudes, wet and dry season temperature variation may be sufficient to make long-term acclimation beneficial. This goes against expectations that tropical species should not show acclimation responses due to the relatively stable temperature environments they inhabit (Ghalambor, 2006), although this was supported in *H. melpomene* where we did not detect any differences in heat knock-down with rearing temperature.

Interestingly we also found evidence of cold acclimation *H. erato*, where individuals reared at cooler temperatures took longer to enter chill coma than those reared at warmer temperatures. This effect has been commonly observed across insect species including some tropical species like *Drosophila melanogaster* (Kristensen et al., 2008) and the butterfly *Bicyclus anynana* (Karl et al., 2014). It is particularly interesting here, given that the cold temperatures used in our knock-out experiments are below anything these butterflies would experience at their capture locations (Montejo-Kovacevich et al., 2020). The distribution of *H. erato* extends beyond the tropics into regions where they could experience extreme cold (Rosser et al., 2012). Thus, it is possible that there is some gene flow from these populations that maintains this plastic response. This could be somewhat similar to the situation in stickleback fish, where genetic variation shared across their broad geographical range has facilitated repeated adaptation to new environments (Reid et al., 2021). We did not observe any cold acclimation effect in *H. melpomene*, consistent with their differing latitudinal ranges.

In temperate butterflies, developmental rates often increase with altitude (Leingärtner et al., 2014) to compensate for shorter seasons and cooler temperatures (Hodkinson, 2005),

although due to differences in voltinism, some species show the opposite trend, with slowed development and one generation per year at higher altitude, and faster development to allow for more generations at lower altitude (Karl et al., 2008). We did not observe any difference in developmental rate between populations in our common garden experiment, suggesting that in tropical systems the lack of seasonality may relax selection on development time, as life-cycles do not need to be completed within a fixed time-window. Nevertheless, *H. melpomene* did show faster larval development in high compared to low altitude populations when reared at 19°C, suggesting that selection has favoured the ability to develop faster at cooler high-altitude temperatures in this species. Faster developmental times may be selected to reduce vulnerability during juvenile stages to predation as well as allowing for earlier dispersal as adults, which could be beneficial when competing for mates and hostplants (Sinclair et al., 2012). A possible explanation for the difference between species is that *H. melpomene* is a host-plant specialist, which may mean that they experience greater resource competition than *H. erato*, which is more generalist.

Many studies report differences in body size with altitude in insects (Hodkinson, 2005; Horne et al., 2018; Montejo-Kovacevich et al., 2019), sometimes following Bergmann's rule (Bergmann, 1848) where warmer environments contain individuals or species with smaller body size. This has been suggested to be adaptive based on larger individuals being more thermally buffered by greater surface-area to volume ratios (Ashton, 2002; Svensson et al., 2023). While this is logical for endotherms, the predictions in ectotherms are less clear and trends found in the wild are likely to be highly biased by differences in growth-rates (Horne et al., 2018; Mousseau, 1997; Svensson et al., 2023). We did not detect any difference in body size between populations when reared under common-garden conditions, suggesting that

previously reported differences in size with altitude in these species are due to plasticity (Montejo-Kovacevich et al., 2019). Indeed, we found a strong effect of rearing temperature on body size that followed the temperature size rule, a common reaction norm where increases in rearing temperature decrease body size (Atkinson, 1994), although a recent meta-analysis suggested that this trend does not hold up across butterfly species (Coradini Schirmer & Malheiros Gawryszewski, 2026). We found this was tightly coupled with development time, where shorter development times (caused by warmer temperatures) also led to smaller pupae. Whether this plasticity provides an adaptive advantage or is just a general biological consequence (Kingsolver & Huey, 2008) has been contested (Teplitsky & Millien, 2014). The effect of body size on thermoregulation in insects appears to be highly variable (Kemp & Krockenberger, 2004; Klockmann et al., 2017). Our results were generally consistent with larger-body sizes being beneficial in cooler temperatures, and so with the observed plasticity being adaptive, as in both species larger individuals were more cold-tolerant and in *H. melpomene* larger individuals were more susceptible to heat, although in *H. erato* there was some evidence that larger individuals were more heat tolerant.

Conclusion

Consistent with Janzen's hypothesis (Janzen, 1967) we do find evidence for adaptive differences between populations of two species of tropical butterflies across a relatively narrow altitude range (~400 m.a.s.l. to ~1200 m.a.s.l.). However, these differences are primarily in morphological traits, principally wing colour and shape. Further work is needed to understand exactly how these traits confer adaptive benefits between populations, but

their high heritability and parallel divergence between these two species, suggests they are likely to be adaptive.

In contrast we find little evidence for fixed differences in physiological and developmental traits between populations, although we do find high heritability for these traits. Lack of divergence in traits with high heritability would suggest that selection is absent or too weak to overcome gene flow across the altitudinal cline (Mallet et al., 1990). Alternatively, selection may be acting in a more complex way on the levels of plasticity in these traits. In support of the latter, we find intriguing genotype-by-environment interactions in thermal tolerance and developmental rate in *H. erato* and *H. melpomene* respectively, highlighting the benefits of reciprocal-rearing experimental designs for teasing apart genetic and environmental effects on traits. The very different responses we find between species in these traits could reflect differences in ecological or evolutionary constraints between the two species and may influence how each is able to respond to changing environments. The variation in heat acclimation that we observe in *H. erato* may be particularly beneficial in helping this species to respond to climate change, while the lack of any acclimation response in *H. melpomene* may make it more vulnerable. Nevertheless, the presence of genetic variation for thermal tolerance in both species, suggests that they have the potential to adapt to changing environments and climate change.

The presence of altitude-associated phenotypic differences is consistent with previous work in these same populations, identifying signatures of adaptive genomic differences (Montejo-Kovacevich et al., 2022). The next step will be to understand how the genetic variation controls the phenotypic variation we have found. This will allow for a deeper understanding

of the adaptive potential of these traits and how they may be influenced by current and future climates.

References

- Ashton, K. G. (2002). Patterns of within-species body size variation of birds: strong evidence for Bergmann's rule. *Global Ecology and Biogeography*, 11(6), 505–523.
<https://doi.org/10.1046/J.1466-822X.2002.00313.X>
- Atkinson, D. (1994). Temperature and Organism Size—A Biological Law for Ectotherms? *Advances in Ecological Research*, 25(C), 1–58. [https://doi.org/10.1016/S0065-2504\(08\)60212-3](https://doi.org/10.1016/S0065-2504(08)60212-3)
- Bainbridge, H. E., Brien, M. N., Morochz, C., Salazar, P. A., Rastas, P., & Nadeau, N. J. (2020). Limited genetic parallels underlie convergent evolution of quantitative pattern variation in mimetic butterflies. *Journal of Evolutionary Biology*, 33(11), 1516–1529.
<https://doi.org/10.1111/JEB.13704>
- Barton, M., & Porter, W. (2014). Behavioural thermoregulation and the relative roles of convection and radiation in a basking butterfly. *Journal of Thermal Biology*, 41(1), 65–71. <https://doi.org/10.1016/j.jtherbio.2014.02.004>
- Bates, D., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, 67(1), 1–48.
<https://doi.org/10.18637/JSS.V067.I01>
- Bergmann, C. (1848). *Über die Verhältnisse der Wärmeökonomie der Thiere zu ihrer Grösse*.
https://books.google.com/books?hl=en&lr=&id=EHo-AAAAcAAJ&oi=fnd&pg=PP3&ots=YI_UpNkcf3&sig=fBHqkKOuH8EB7c7sqR2pFucv-w4

- Bogert, C. M. (1949). Thermoregulation in Reptiles, A Factor in Evolution. *Evolution*, 3(3), 195. <https://doi.org/10.2307/2405558>
- Brakefield, P. M., & Kesbeke, F. (1997). Genotype-environment interactions for insect growth in constant and fluctuating temperature regimes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 264(1382), 717–723. <https://doi.org/10.1098/RSPB.1997.0102>
- Brehm, G., Zeuss, D., & Colwell, R. K. (2019). Moth body size increases with elevation along a complete tropical elevational gradient for two hyperdiverse clades. *Ecography*, 42(4), 632–642. <https://doi.org/10.1111/ECOG.03917>
- Burnham, K. P., & Anderson, D. R. (2004). *Model selection and multimodel inference* (A practica).
- Clusella Trullas, S., van Wyk, J. H., & Spotila, J. R. (2007). Thermal melanism in ectotherms. *Journal of Thermal Biology*, 32(5), 235–245. <https://doi.org/10.1016/J.JTHERBIO.2007.01.013>
- Coradini Schirmer, S., & Malheiros Gawryszewski, F. (2026). Developmental plasticity of butterflies: A meta-analysis of temperature effects. *Journal of Animal Ecology*, 95(4), 618–632. <https://doi.org/10.1111/1365-2656.70217>
- Dasmahapatra, K. K., Walters, J. R., Briscoe, A. D., Davey, J. W., Whibley, A., Nadeau, N. J., Zimin, A. V., Salazar, C., Ferguson, L. C., Martin, S. H., Lewis, J. J., Adler, S., Ahn, S. J., Baker, D. A., Baxter, S. W., Chamberlain, N. L., Ritika, C., Counterman, B. A., Dalmay, T., ... Jiggins, C. D. (2012). Butterfly genome reveals promiscuous exchange of mimicry adaptations among species. *Nature*, 487(7405), 94–98. <https://doi.org/10.1038/nature11041>
- Dufour, P. C., Willmott, K. R., Padrón, P. S., Xing, S., Bonebrake, T. C., & Scheffers, B. R.

- (2018). Divergent melanism strategies in Andean butterfly communities structure diversity patterns and climate responses. *Journal of Biogeography*, 45(11), 2471–2482. <https://doi.org/10.1111/JBI.13433>
- Ellers, J., & Boggs, C. L. (2002). The evolution of wing color in *Colias* butterflies: heritability, sex linkage, and population divergence. *Evolution; International Journal of Organic Evolution*, 56(4), 836–840. <https://doi.org/10.1111/J.0014-3820.2002.TB01394.X>
- Ellers, J., & Boggs, C. L. (2004). Functional ecological implications of intraspecific differences in wing melanization in *Colias* butterflies. *Biological Journal of the Linnean Society*, 82(1), 79–87. <https://doi.org/10.1111/J.1095-8312.2004.00319.X>
- Falconer, D. S., & Mackay, T. F. . (1996). *Introduction to Quantitative Genetics* (4th ed.). Pearson.
- Fischer, K., Dierks, A., Franke, K., Geister, T. L., Liszka, M., Winter, S., & Pflicke, C. (2010). Environmental Effects on Temperature Stress Resistance in the Tropical Butterfly *Bicyclus Anynana*. *PLOS ONE*, 5(12), e15284. <https://doi.org/10.1371/JOURNAL.PONE.0015284>
- Fox, R. J., Donelson, J. M., Schunter, C., Ravasi, T., & Gaitán-Espitia, J. D. (2019). Beyond buying time: the role of plasticity in phenotypic adaptation to rapid environmental change. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 374(1768). <https://doi.org/10.1098/RSTB.2018.0174>
- Franke, K., Karl, L., Centeno, T. P., Feldmeyer, B., Lassek, C., Oostra, V., Riedel, K., Stanke, M., Wheat, C. W., & Fischer, K. (2019). Effects of adult temperature on gene expression in a butterfly: Identifying pathways associated with thermal acclimation. *BMC Evolutionary Biology*, 19(1), 1–16. <https://doi.org/10.1186/S12862-019-1362-Y/FIGURES/3>
- Ghalambor, C. K. (2006). Are mountain passes higher in the tropics? janzen's hypothesis

revisited. *Integrative and Comparative Biology*, 46(1), 5–17.

<https://doi.org/10.1093/icb/icj003>

Gienapp, P., Teplitsky, C., Alho, J. S., Mills, J. A., & Merilä, J. (2008). Climate change and evolution: disentangling environmental and genetic responses. *Molecular Ecology*, 17(1), 167–178. <https://doi.org/10.1111/J.1365-294X.2007.03413.X>

González-Tokman, D., Córdoba-Aguilar, A., Dáttilo, W., Lira-Noriega, A., Sánchez-Guillén, R. A., & Villalobos, F. (2020). Insect responses to heat: physiological mechanisms, evolution and ecological implications in a warming world. *Biological Reviews*, 95(3), 802–821. <https://doi.org/10.1111/BRV.12588>

Günter, F., Beaulieu, M., Brunetti, M., Lange, L., Schmitz Ornés, A., & Fischer, K. (2019). Latitudinal and altitudinal variation in ecologically important traits in a widespread butterfly. *Biological Journal of the Linnean Society*, 128(3), 742–755. <https://doi.org/10.1093/biolinnean/blz133>

Guppy, C. S. (1986). Geographic variation in wing melanism of the butterfly *Parnassius phoebus* F. (Lepidoptera: Papilionidae). *Canadian Journal of Zoology*, 64(4), 956–962. <https://doi.org/10.1139/Z86-145>

Hijmans, R. J. (2023). *Geographic Data Analysis and Modeling [R package raster version 3.6-26]*. <https://cran.r-project.org/package=raster>

Hodkinson, I. D. (2005). Terrestrial insects along elevation gradients: species and community responses to altitude. *Biological Reviews*, 80(3), 489–513. <https://doi.org/10.1017/S1464793105006767>

Horne, C. R., Hirst, A. G., & Atkinson, D. (2018). Insect temperature–body size trends common to laboratory, latitudinal and seasonal gradients are not found across altitudes. *Functional Ecology*, 32(4), 948–957. <https://doi.org/10.1111/1365->

- Humboldt, A. von, & Bonpland, A. (1807). *Essay on the Geography of Plants*.
- Janzen, D. H. (1967). Why Mountain Passes are Higher in the Tropics. *The American Naturalist*, 101(919), 233–249. <https://doi.org/10.1086/282487>
- Jiggins, C. D. (2017). *The Ecology and Evolution of Heliconius Butterflies* (1st ed.). OUP Oxford. <https://doi.org/10.1093/acprof:oso/9780199566570.001.0001>
- Karl, I., Becker, M., Hinzke, T., Mielke, M., Schiffler, M., & Fischer, K. (2014). Interactive effects of acclimation temperature and short-term stress exposure on resistance traits in the butterfly *Bicyclus anynana*. *Physiological Entomology*, 39(3), 222–228. <https://doi.org/10.1111/PHEN.12065>
- Karl, I., Janowitz, S. A., Fischer, K., Karl, I., Janowitz, S. A., & Fischer, K. (2008). Altitudinal life-history variation and thermal adaptation in the copper butterfly *Lycaena tityrus*. *Oikos*, 117(5), 778–788. <https://doi.org/10.1111/J.0030-1299.2008.16522.X>
- Kemp, D. J., & Krockenberger, A. K. (2004). Behavioural thermoregulation in butterflies: the interacting effects of body size and basking posture in *Hypolimnys bolina* (L.) (Lepidoptera : Nymphalidae). *Australian Journal of Zoology*, 52(3), 229–239. <https://doi.org/10.1071/ZO03043>
- Kingsolver, J. G., & Buckley, L. B. (2017). Evolution of plasticity and adaptive responses to climate change along climate gradients. *Proceedings of the Royal Society B: Biological Sciences*, 284(1860). <https://doi.org/10.1098/RSPB.2017.0386>
- Kingsolver, J. G., & Huey, R. B. (2008). Size, temperature, and fitness: Three rules. *Evolutionary Ecology Research*, 10(2), 251–268.
- Klein, A. L., & de Araújo, A. M. (2013). Sexual Size Dimorphism in the Color Pattern Elements of Two Mimetic *Heliconius* Butterflies. *Neotropical Entomology*, 42(6), 600–606.

<https://doi.org/10.1007/S13744-013-0157-X/TABLES/4>

Klockmann, M., Günter, F., & Fischer, K. (2017). Heat resistance throughout ontogeny: body size constrains thermal tolerance. *Global Change Biology*, 23(2), 686–696.

<https://doi.org/10.1111/GCB.13407>

Körner, C. (2007). The use of “altitude” in ecological research. *Trends in Ecology and Evolution*, 22(11), 569–574. <https://doi.org/10.1016/j.tree.2007.09.006>

Kristensen, T. N., Hoffmann, A. A., Overgaard, J., Sørensen, J. G., Hallas, R., & Loeschcke, V. (2008). Costs and benefits of cold acclimation in field-released *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, 105(1), 216–221.

https://doi.org/10.1073/PNAS.0708074105/SUPPL_FILE/08074FIG3.PDF

Kuyucu, A. C., Sahin, M. K., & Caglar, S. S. (2018). The relation between melanism and thermal biology in a colour polymorphic bush cricket, *Isophya rizeensis*. *Journal of Thermal Biology*, 71, 212–220. <https://doi.org/10.1016/J.JTHERBIO.2017.11.017>

Ladwa, S., Salazar-Carrion, P., Guachamin-Rosero, M., Gavilanes, K., Bacquet, C. N., & Nadeau, N. (2026). *Altitude and canopy-cover shape passive heat gain and active thermoregulation in tropical butterflies*.

<https://doi.org/10.22541/AUTHOREA.15003143/V1>

Leingärtner, A., Krauss, J., & Steffan-Dewenter, I. (2014). Species richness and trait composition of butterfly assemblages change along an altitudinal gradient. *Oecologia*, 175(2), 613–623. <https://doi.org/10.1007/S00442-014-2917-7/TABLES/3>

Leroi, A. M., Bennett, A. F., & Lenski, R. E. (1994). Temperature acclimation and competitive fitness: an experimental test of the beneficial acclimation assumption. *Proceedings of the National Academy of Sciences of the United States of America*, 91(5), 1917.

<https://doi.org/10.1073/PNAS.91.5.1917>

Mallet, J., Barton, N., Gerardo Lamas, M., Jose Santisteban, C., Manuel Muedas, M., & Eeley,

H. (1990). Estimates of selection and gene flow from measures of cline width and linkage disequilibrium in *heliconius* hybrid zones. *Genetics*, 124(4), 921–936.

<https://doi.org/10.1093/GENETICS/124.4.921>

Mallet, J., & Barton, N. H. (1989). Strong natural Selection in a warning-color hybrid zone.

Evolution, 43(2), 421–431. <https://doi.org/10.1111/J.1558-5646.1989.TB04237.X>

Massicotte, P., & South, A. (2023). *rnaturalearth: World Map Data from Natural Earth*.

<https://docs.ropensci.org/rnaturalearth/>,%0Ahttps://github.com/ropensci/rnaturalearth

Mazerolle, M. J. (2023). *CRAN - Package AICcmodavg*. [https://cran.r-](https://cran.r-project.org/web/packages/AICcmodavg/index.html)

[project.org/web/packages/AICcmodavg/index.html](https://cran.r-project.org/web/packages/AICcmodavg/index.html)

Montejo-Kovacevich, G., Martin, S. H., Meier, J. I., Bacquet, C. N., Monllor, M., Jiggins, C. D.,

& Nadeau, N. J. (2020). Microclimate buffering and thermal tolerance across elevations in a tropical butterfly. *Journal of Experimental Biology*, 223(8).

<https://doi.org/10.1242/jeb.220426>

Montejo-Kovacevich, G., Meier, J. I., Bacquet, C. N., Warren, I. A., Chan, Y. F., Kucka, M.,

Salazar, C., Rueda-M, N., Montgomery, S. H., McMillan, W. O., Kozak, K. M., Nadeau, N.

J., Martin, S. H., & Jiggins, C. D. (2022). Repeated genetic adaptation to altitude in two tropical butterflies. *Nature Communications* 2022 13:1, 13(1), 1–16.

<https://doi.org/10.1038/s41467-022-32316-x>

Montejo-Kovacevich, G., Salazar, P. A., Smith, S. H., Gavilanes, K., Bacquet, C. N., Chan, Y. F.,

Jiggins, C. D., Meier, J. I., & Nadeau, N. J. (2021). Genomics of altitude-associated wing shape in two tropical butterflies. *Molecular Ecology*, 30(23), 6387–6402.

<https://doi.org/10.1111/MEC.16067>

- Montejo-Kovacevich, G., Smith, J. E., Meier, J. I., Bacquet, C. N., Whiltshire-Romero, E., Nadeau, N. J., & Jiggins, C. D. (2019). Altitude and life-history shape the evolution of *Heliconius* wings. *Evolution*, 73(12), 2436–2450. <https://doi.org/10.1111/evo.13865>
- Moore, M. P., Whiteman, H. H., & Martin, R. A. (2019). A mother's legacy: the strength of maternal effects in animal populations. *Ecology Letters*, 22(10), 1620–1628. <https://doi.org/10.1111/ELE.13351>
- Mousseau, T. A. (1997). Ectotherms Follow the Converse to Bergmann's Rule. *Evolution*, 51(2), 630. <https://doi.org/10.2307/2411138>
- Murren, C. J., Auld, J. R., Callahan, H., Ghalambor, C. K., Handelsman, C. A., Heskell, M. A., Kingsolver, J. G., Maclean, H. J., Masel, J., Maughan, H., Pfennig, D. W., Relyea, R. A., Seiter, S., Snell-Rood, E., Steiner, U. K., & Schlichting, C. D. (2015). Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. *Heredity* 2015 115:4, 115(4), 293–301. <https://doi.org/10.1038/hdy.2015.8>
- Nadeau, N. J. (2016). Genes controlling mimetic colour pattern variation in butterflies. *Current Opinion in Insect Science*, 17, 24–31. <https://doi.org/10.1016/J.COIS.2016.05.013>
- Nakagawa, S., & Schielzeth, H. (2010). Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. *Biological Reviews of the Cambridge Philosophical Society*, 85(4), 935–956. <https://doi.org/10.1111/J.1469-185X.2010.00141.X>
- Nève, G., & Després, L. (2020). Cold adaptation across the elevation gradient in an alpine butterfly species complex. *Ecological Entomology*, 45(5), 997–1003. <https://doi.org/10.1111/EEN.12875>
- Niitepõld, K. (2010). Genotype by temperature interactions in the metabolic rate of the Glanville fritillary butterfly. *Journal of Experimental Biology*, 213(7), 1042–1048.

<https://doi.org/10.1242/JEB.034132>

Pebesma, E. (2018). Simple features for R: Standardized support for spatial vector data. *R Journal*, 10(1), 439–446. <https://doi.org/10.32614/RJ-2018-009>

Pebesma, E., & Bivand, R. (2023). Spatial Data Science: With Applications in R. *Spatial Data Science: With Applications in R*, 1–300. <https://doi.org/10.1201/9780429459016>

Reid, K., Bell, M. A., & Veeramah, K. R. (2021). Threespine Stickleback: A Model System for Evolutionary Genomics. *Annual Review of Genomics and Human Genetics*, 22(Volume 22, 2021), 357–383. <https://doi.org/10.1146/ANNUREV-GENOM-111720-081402/CITE/REFWORKS>

Roland, J. (1982). Melanism and diel activity of alpine Colias (Lepidoptera: Pieridae). *Oecologia*, 53(2), 214–221. <https://doi.org/10.1007/BF00545666/METRICS>

Rosa, E., & Saastamoinen, M. (2020). Beyond thermal melanism: association of wing melanization with fitness and flight behaviour in a butterfly. *Animal Behaviour*, 167, 275–288. <https://doi.org/10.1016/J.ANBEHAV.2020.07.015>

Rosser, N., Phillimore, A. B., Huertas, B., Willmott, K. R., & Mallet, J. (2012). Testing historical explanations for gradients in species richness in heliconiine butterflies of tropical America. *Biological Journal of the Linnean Society*, 105(3), 479–497. <https://doi.org/10.1111/J.1095-8312.2011.01814.X>

Scharf, I., Sbilordo, S. H., & Martin, O. Y. (2014). Cold tolerance in flour beetle species differing in body size and selection temperature. *Physiological Entomology*, 39(1), 80–87. <https://doi.org/10.1111/PHEN.12050>

Searle, S. R., Speed, F. M., & Milliken, G. A. (2023). Estimated Marginal Means, aka Least-Squares Means [R package emmeans version 1.8.9]. *American Statistician*, 34(4), 216–221. <https://doi.org/10.1080/00031305.1980.10483031>

- Sejerkilde, M., Sørensen, J. G., & Loeschcke, V. (2003). Effects of cold- and heat hardening on thermal resistance in *Drosophila melanogaster*. *Journal of Insect Physiology*, 49(8), 719–726. [https://doi.org/10.1016/S0022-1910\(03\)00095-7](https://doi.org/10.1016/S0022-1910(03)00095-7)
- Semper, C. (1883). *The natural conditions of existence as they affect animal life*. https://books.google.com/books?hl=en&lr=&id=6jUaAAAAYAAJ&oi=fnd&pg=PA1&ots=FMaCUP_s4w&sig=MUK6r5_PhIz4mYF4NY6TtWhG64s
- Sinclair, B. J., Williams, C. M., & Terblanche, J. S. (2012). Variation in Thermal Performance among Insect Populations. *Physiological and Biochemical Zoology*, 85(6), 594–606. <https://doi.org/10.1086/665388>
- Slade, E. M., & Ong, X. R. (2023). The future of tropical insect diversity: strategies to fill data and knowledge gaps. *Current Opinion in Insect Science*, 58, 101063. <https://doi.org/10.1016/J.COIS.2023.101063>
- Slowikowski, K. (2023). *Automatically Position Non-Overlapping Text Labels with “ggplot2” [R package ggrepel version 0.9.4]*. <https://cran.r-project.org/package=ggrepel>
- Stoffel, M. A., Nakagawa, S., & Schielzeth, H. (2017). rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. *Methods in Ecology and Evolution*, 8(11), 1639–1644. <https://doi.org/10.1111/2041-210X.12797>
- Sullivan, J. B., & Miller, W. E. (2007). Intraspecific body size variation in macrolepidoptera as related to altitude of capture site and seasonal generation. *Journal of the Lepidopterists' Society*, 61(2), 72–77. [http://images.peabody.yale.edu/lepsoc/jls/2000s/2007/2007\(2\)72-Sullivan.pdf](http://images.peabody.yale.edu/lepsoc/jls/2000s/2007/2007(2)72-Sullivan.pdf)
- Svensson, E. I., Gómez-Llano, M., & Waller, J. T. (2023). Out of the tropics: Macroevolutionary size trends in an old insect order are shaped by temperature and predators. *Journal of Biogeography*, 50(3), 489–502. <https://doi.org/10.1111/JBI.14544>

Teplitsky, C., & Millien, V. (2014). Climate warming and Bergmann's rule through time: is there any evidence? *Evolutionary Applications*, 7(1), 156.

<https://doi.org/10.1111/EVA.12129>

Walters, J. R., Corbins, C., Hardcastle, T. J., & Jiggins, C. D. (2012). Evaluating female remating rates in light of spermatophore degradation in *Heliconius* butterflies: pupal-mating monandry versus adult-mating polyandry. *Ecological Entomology*, 37(4), 257–268. <https://doi.org/10.1111/J.1365-2311.2012.01360.X>

Weller, H. I., & Westneat, M. W. (2019). Quantitative color profiling of digital images with earth mover's distance using the R package colordistance. *PeerJ*, 7(2).

<https://doi.org/10.7717/PEERJ.6398>

Wickham, H. (2016). ggplot2 Elegant Graphics for Data Analysis. *Use R! Series*, 211.

Wilson, R. S., & Franklin, C. E. (2002). Testing the beneficial acclimation hypothesis. *Trends in Ecology and Evolution*, 17(2), 66–70. [https://doi.org/10.1016/S0169-5347\(01\)02384-9](https://doi.org/10.1016/S0169-5347(01)02384-9)