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Violaine Ossola

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Violaine Ossola. Thermal adaptation in tropical clearwing butterflies. Life Sciences [q-bio]. Université Paris Cité, 2023. English. NNT: . tel-04483387v2

HAL Id: tel-04483387

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Université Paris Cité
Ecole Doctorale 474 - Frontière de l'Innovation en Recherche et Education

Thèse d'écologie, évolution et biologie environnementale

Thermal adaptation in tropical clearwing butterflies

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Soutenue le 14 décembre 2023

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General Introduction

1. Animal thermoregulation

1.1. A spectrum from ectothermy to endothermy

Every living organism has an optimal temperature range in which cell function and energy expenditure are optimized. In animals, endothermy, is a thermoregulatory strategy of high metabolic rate enabling sufficient body heat to maintain activity over a wide range of external temperatures. Stable endothermy, or homeothermy, which has evolved in a convergent way in birds and mammals, provide them with a relatively constant body temperature and open the possibility of temporal and spatial niches inaccessible to strict ectotherms (Angilletta, 2009). On the other hand, ectothermy, found in most fish, reptiles, amphibians and invertebrates, is a metabolic strategy in which the body temperature is a function of the conditions of the environment.

The body temperature of an ectotherm is the result of energy transfers between the individual and its surrounding, such as conduction, convection, evaporation, and radiative flows. In practice, there is a full spectrum of thermal strategies used by animals, from strict thermoregulation, when body temperature is absolutely regulated to a constant level regardless of external conditions, to virtually total thermal passivity - or thermoconformity -, when temperature is perfectly linked to that of the immediate environment and its various parameters such as the rate of light radiation and air movements (Huey and Slatkin, 1976) (Fig1).

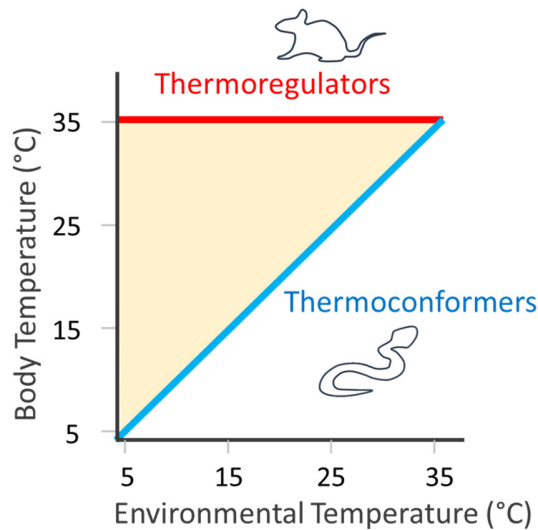


Figure 1. This graph, adapted from (Huey and Slatkin, 1976), shows body temperature as a function of environmental temperature (often simplified as air temperature). When an organism is said to be a thermoconformer, its body temperature is perfectly equivalent to that of its immediate environment. When an organism is capable of adjusting its internal temperature whatever the environmental conditions, it is said to be a thermoregulator. There is a whole spectrum of possible body temperature regulation strategies between strict thermoconformism and perfectly stable temperature.

Thermoregulation involves any kind of physiological, morphological, or behavioral mechanisms that enable an organism to modulate its body temperature (Angilletta, 2009). Some ectotherms are capable, to a lesser extent, of producing internal heat by means of a high metabolic rate (Heinrich, 1974), which enables them to be active at unfavorable times. This is the case in some flying insects such as for example dragonflies, moths and bumblebees. These insects need to produce heat in preparation for flight by increasing their thoracic muscular activity, where the locomotor muscles are located (Heinrich, 1972, 1995).

Increasing body temperature is costly for ectotherms, and requires a high amount of energy. Cold conditions are therefore associated with reduced activity as metabolic processes slow down. (Buckley and Jetz, 2010; Dillon et al., 2010). However, the metabolic needs of ectotherms to maintain activity are up to 10 times lower than those of endotherms, with the possibility of surviving periods of scarce resources without nutrient reserve for a longer time, unlike endotherms, which need to maintain a high energy expenditure to cope with the cold (Pough, 1980; Porter, 2002; Koteja, 2004). In addition, high metabolic activity involves the risk of overheating (Norris and Kunz, 2012). For instance, due to high heat production in flight, some migratory birds of the genus *Somateria* have to reduce their flight time to avoid hyperthermia (Guillemette et al., 2016). Similarly, in the beetle *Scarabaeus sacer*, the heat generated by thoracic muscles during flight increases the danger of overheating in warm ambient conditions (Verdú et al., 2012).

1.2. Thermal requirements and performances

Thermal performance curves define the relationship between a trait, such as locomotion, growth rate or overall fitness, as a function of body temperature (Huey and Slatkin, 1976; Huey and Stevenson, 1979). They reflect how thermally restricted the activity of an organism is, and give an insight into how performance varies with environmental thermal variations (Fig 2). In diurnal Lepidoptera, the biological model of this thesis, internal body temperature has direct implications in many aspects of their ecology, and therefore fitness-related traits, such as locomotor performance, predator avoidance, mate location, foraging, oviposition, and dispersal (Watt, 1968; Kingsolver, 1983; Heinrich, 1993; Van Dyck et al., 1997; Kemp and Krockenberger, 2002).

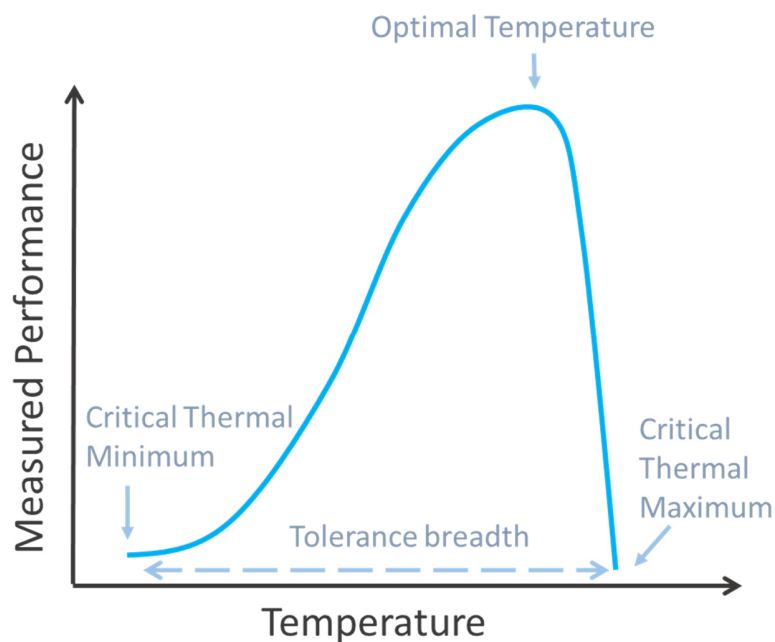


Figure 2. A standard thermal performance curve. Thermal performance curves are efficient tools to describe the relationship between the performance of a trait and the body temperature (Huey and Stevenson, 1979; Angilletta, 2009). As body temperature increases, performance reaches an optimum, and drops at high temperatures. Above and below critical temperatures, performance is reduced completely to a critical point.

Thermal requirement and performance measurements give us an idea of the fundamental niches and global tolerances of species and populations, as well as their resilience, a valuable information in the context of climatic change. The most immediately visible performances in response to temperature in insects are locomotor abilities. Locomotor abilities diminish as body temperature moves away from its temperature optimum, as described in temperature performance curves.

To assess thermal tolerance, it is possible to test individuals' tolerance to heat and cold. With some variations in protocols, two main methods are frequently used in research on ectotherm thermal tolerance : dynamic and static assays. Dynamic assays involve exposing individuals to a progressive temperature gradient, until a critical point is reached, at which the individual loses locomotor performance (Gallego et al., 2016; Ashe-Jepson et al., 2023). The trial often lasts several hours, providing information on the critical temperatures above or below which injury accumulation makes activity impossible. These long trials come with the risk of starvation and desiccation during the experiment. Static assays, on the other hand, expose individuals abruptly to extreme temperatures (Ashoff and Schmitt, 2014; Montejó-Kovacevich et al., 2020). The trial is shorter, often lasting less than an hour. The outcome measured is the time until the loss of locomotion, or the time until full recovery of locomotor function once the exposure to temperature stress has ended. The results obtained using both methods are highly correlated and give comparable conclusions on heat tolerance across *Drosophila* species (Jørgensen et al., 2019) and static assays may prove superior to measure small differences in thermal tolerance (Winther Bak et al., 2020). Even if it is difficult to compare the thermal stress experienced during the tests to that of natural stress conditions endured in fluctuating and unpredictable environments, both methods are reliable protocols for inter-individual tolerance comparisons (Jørgensen et al., 2021).

2. Thermal control in ectotherms

Although they have less internal control of their body temperature than endotherms, ectotherms use various morphological, physiological and behavioral strategies to buffer their body temperature in a given direction.

2.1. Effect of radiation and colors on animal temperature regulation

According to the law of energy conservation, for each wavelength λ , absorption, transmission and reflection sum up to the incident light as $1 = \text{Absorption}(\lambda) + \text{Transmission}(\lambda) + \text{Reflection}(\lambda)$. The light absorbed by a material is transformed into thermal energy, resulting in heating. Then, knowing the absorption properties of a body surface gives valuable information about its thermal capacities. The spectrum of solar radiation reaching the earth's surface accounts for around 48% of ultraviolet (UV) (~ 300 to $\sim 400\text{nm}$) and human-visible light (~ 400 to $\sim 700\text{nm}$), and up to 52% in the near-infrared (NIR) (~ 700 to $\sim 2500\text{nm}$) (Fig 3). The latter wavelengths are therefore the largest contributor to the thermal heating of a surface.

Not visible to animals, the optical properties of a body in the NIR are therefore mainly the result of thermal adaptation, unlike UV and visible radiation, also involved in other aspects of species ecology, such as intra and interspecific signaling or camouflage (Nussear et al., 2000; Stuart-Fox et al., 2017). NIR reflectance was found to be well predicted by climatic conditions

in large butterfly assemblages (Munro et al., 2019; Kang et al., 2021). These conclusions were established by the description of full specimen surface reflection, using measurements on photos taken using sensors sensitive to different wavelengths (Fig 3). Finally, all the elements of an environment, such as animals, stones, vegetation, and even the atmosphere, generate thermal radiation in the far infrared range, mostly between 8 and 12 μm , a process directly related to their temperature. A body's ability to absorb and emit these wavelengths depends mainly on its intrinsic structural properties, which affect its thermal energy budget (Norris, 1967; Porter and Gates, 1969).

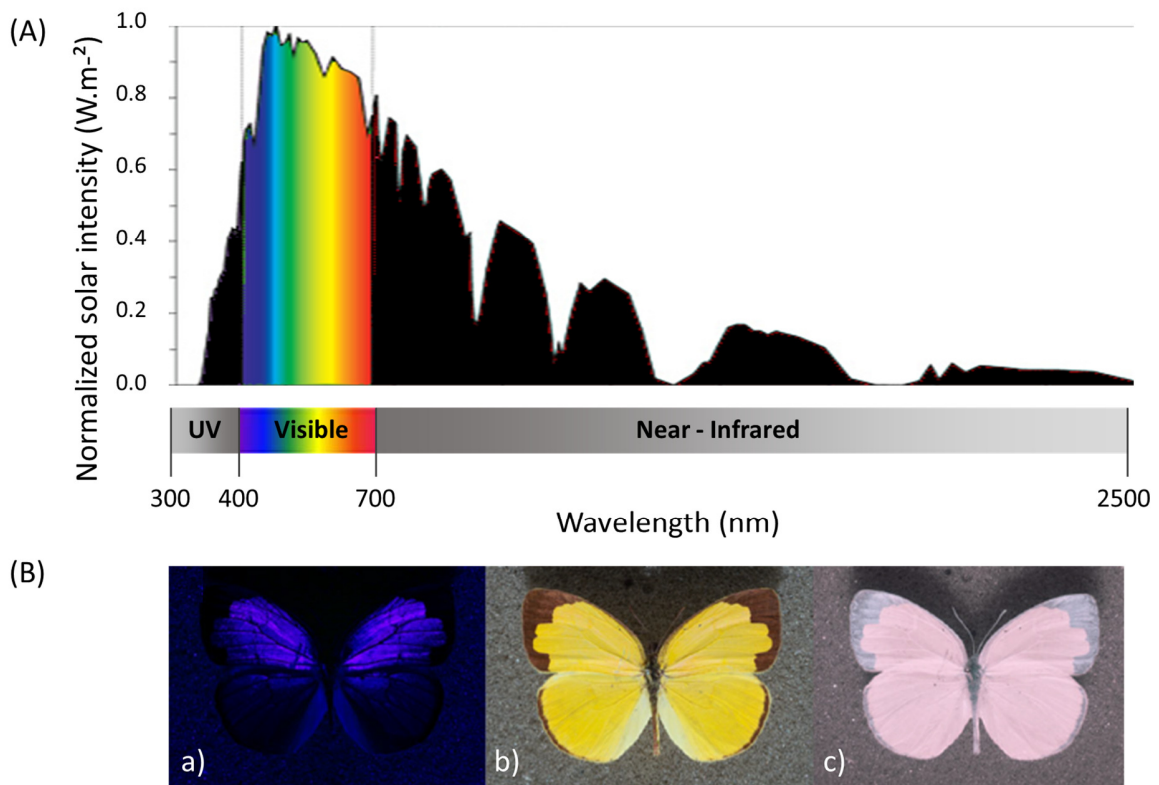


Figure 3. A) The standard irradiance of the solar spectrum reaching the earth at ground level shows that the ultraviolet (UV) range (roughly 300 to 400 nm) represent around 5% of the total solar energy distribution, human-visible wavelengths (400 to 700 nm) 43% and near-infrared (NIR) (700 to 2500 nm) around 52%. B) Photos in false colors from Munro et al. (2019), taken with different filters, shows that the same butterfly have divergent patterns of light reflection in the a) UV, b) visible and c) NIR wavelengths. Lighter colors indicate higher reflectance.

The color of a surface has a direct influence on its radiation absorption. Quite instinctively, the darker the surface, the more light it absorbs and the hotter it gets, because the energy absorbed is converted into heat. In butterflies, it has long been known that wings play a vital role in increasing body temperature (Wasserthal, 1975), and that their degree of darkness -referred as melanism- is positively correlated to heat absorption (Watt, 1968;

Schmitz, 1994). The degree of melanization is therefore linked to thermal performance and activity and has been shown to be correlated with flight capabilities in diurnal butterflies belonging to the families Pieridae, Papilionidae and Nymphalidae, with implications for periods of activity, predator deflection and mating success (Guppy, 1986; Roland, 2006; Davis et al., 2012). Using infrared cameras, it is now possible to determine with great precision which colors on the wings are the most important in the overall heating of the butterfly, and to make a direct link between the degree of melanization and thermal properties (Liao et al., 2019; Krishna et al., 2020). Its influence on fitness-related traits makes coloration an efficient adaptive response to temperature conditions (Clusella Trullas et al., 2007), and the degree of melanization is an important driver of the body temperature actually reached by insects given the ambient environment.

The absorption properties of the wings may sometimes not be sufficient to modulate temperatures, as they also depend on the size of the individuals or the convection movements of the surrounding air (De Keyser et al., 2015). Along with coloration, body mass has a major influence on an individual's thermal capacity. For instance, larger organisms reach a higher temperature, take longer to reach thermal equilibrium, but maintain it longer than smaller organisms (Porter and Gates, 1969). Their low convective coefficient means they absorb a larger part of solar radiation. To avoid overheating, large ectotherms may then need greater skin reflectance to decrease heat load. On the other hand, small ectotherms have higher surface-to-mass ratios and greater convective heat transfer coefficients. Consequently, small ectotherms like small lizards can afford to be melanistic without much overheating consequence, as they can dissipate heat rapidly thanks to convective cooling (Norris, 1967).

2.2. Behavioral and physiological thermoregulation

Most ectotherms rely on behavioral thermoregulation to maintain or adjust their body temperature. They can therefore considerably limit the repercussions of acute temperature variations, and limit their exposure to thermal extremes by maintaining themselves in a narrow temperature range which includes their physiological optimum (Martin and Huey, 2008). This can involve modifying posture, or increasing the surface area for heat exchange with the environment. Basking is a very common behavior in butterflies (Clench, 1966; Barton et al., 2014), and it involves orientating the wings towards the sun to collect light rays, or using the contact substrate to conduct heat. To avoid overheating, on the other hand, they can also move into the shade or minimize the exposure of their wings to the sun (Rawlins, 1980; Tsai et al., 2020).

Spatial heterogeneity offers efficient refuges for buffering temperature. The choice of microhabitats, in a more or less open environment, in or above the understory or even under the leaves of a plant, can constitute more favorable conditions with minimal energy expenditure to thermoregulate (Kleckova et al., 2014; Pincebourde and Suppo, 2016; Montejo-Kovacevich et al., 2019). Moreover, the period of activity can vary during the day, along with the intensity of sunlight. In Australian butterfly assemblages, lighter, smaller butterflies are

more active in the middle of the day, while darker, larger individuals are more active during the crepuscular hours (Xing et al., 2016). In addition, in some species, physiological adaptations help to limit the impact of thermal stress, through heat-shock proteins or cryoprotectants, especially in harsh environments (Vrba et al., 2017). Plus, many insects are also able to regulate their internal temperature via heat generation by rapid muscle contraction (Heinrich and Pantle, 1975). Thus, ectotherms possess a wide range of behavioral and physiological adjustments to regulate their temperature. It has been suggested that the morphological, physiological and behavioral characters linked to thermoregulation have evolved jointly (Angilletta et al., 2006). It is difficult to predict which adaptive strategies evolve according to a specific environment, as responses depend on the cost-benefit balance given temperature variation through time and space, but also in the face of other selection pressures, such as sexual interactions, competition and predation.

3. Morphological and physiological responses to climatic conditions along ecological gradients

3.1. Variation of climatic conditions along altitudinal gradients

Studying trait variation along environmental gradients informs on the adaptive responses of species and populations to climatic conditions. Latitudinal gradients are generally characterized by a variation in conditions over a scale of several hundred kilometers, whereas elevation represent a homogeneous variation in climate over short horizontal distances. For the latter, this implies negligible variation in other parameters such as day length or seasonality, making mountain systems particularly interesting for the study of climate related traits. Mountain regions are in many cases biodiversity hotspots, especially for butterflies, remarkably diversified in the Andes (Chazot et al., 2019; Doré et al., 2022).

Among abiotic factors, temperature is undoubtedly the one that shows the greatest spatial and temporal fluctuations. Temperature changes at an average rate of about 0.5 to 0.6°C per 100 meters of altitude (Osborne, 2012) and is the parameter that most directly affects the distribution of ectotherms (Dillon, 2006). In addition, atmospheric pressure and oxygen levels decrease uniformly with elevation. They affect breathing and metabolic functioning. An extra metabolic effort is even required if oxygen levels are too low for optimum performance (Hodkinson, 2005). UV radiation, known to increase the risk of mutation and impair cellular processes, is thought to be more important at higher elevation (Beckmann et al., 2014), but the greater cloud cover in altitude can mitigate UV irradiation (Körner, 2007). Unlike temperature, precipitations, wind and seasonality do not vary consistently with altitude but are more specific to the broader climatic zone, with regions at lower latitudes undergoing lower climatic variability and higher annual precipitations (Deutsch et al., 2008).

In addition to being home to a great number of plant and animal species, tropics harbor the highest levels of genetic and phylogenetic diversity in genomes and clades of clades of the major vertebrates and invertebrates taxa (Brown, 2013). In 1967, Janzen investigated the relationship between latitudinal climatic variability and species diversity along elevation gradients. He proposed that the lower seasonal variation in tropical temperatures selected narrower thermal niches and more limited altitudinal dispersion, in contrast to temperate regions where high climatic variability should select wider thermal tolerances. Under this hypothesis, altitudinal ranges are smaller for tropical species because they have shaped narrow thermal tolerance, which leads to high speciation. He hence comments that “mountain passes are higher in the tropics”, meaning that vertical dispersal abilities are weak. Janzen's predictions have received some support: the altitudinal range of vertebrate ectotherms is widest at high latitudes, while tropical vertebrate ectotherms have a narrower altitudinal range (McCain, 2009). Likewise, in arthropods the thermal niche has been shown to be narrower in the tropics than in temperate regions (Polato et al., 2018). As a result, climate warming, although predicted to be of smaller magnitude than in temperate regions, is likely to have the most deleterious consequences on tropical species which are very sensitive to temperature change and are already living close to their optimal temperature (Williams et al., 2007; Deutsch et al., 2008).

3.2. Physiological adaptation in response to temperature conditions

Since their physiological performance depends directly on temperature, ectotherms are strongly affected by climatic fluctuations. They are constrained in their activity, particularly in cold climates, i.e. at high altitudes and latitudes, and thermal limits typically follow a climatic pattern (Clusella-Trullas et al., 2011; Sunday et al., 2011). The description of physiological capacities allows direct comparisons between individuals, populations, species, or communities, as well as the response of these groups to past or future variations (Sinclair et al., 2012, 2016). In high latitudes, ectotherms experience greater seasonal variability and are therefore exposed to a wider range of temperatures. Their thermal minimum is lower than that of species inhabiting tropical climates, where the warm weather is annually more stable (Clusella-Trullas et al., 2011). This trend is asymmetrical since upper thermal limits vary little with latitude (Addo-Bediako et al., 2000; Sunday et al., 2011). Although studies on tolerance along tropical elevation gradients are scarce, similar trends as those found with latitude have been described for altitude : in terrestrial and freshwater ectotherms, thermal minimum decline towards higher elevations, while upper maximum do not vary (Sunday et al., 2019).

Thermal performance can be sex- and size-dependent (Dillon et al., 2009; Oyen et al., 2016), and tuned by plastic responses and adaptation (Kefford et al., 2022). Warm temperature exposure may, for example, shift the thermal optimum of ectotherms to higher temperatures while reducing it at low temperatures (Gilchrist et al., 1997), increase performance and fitness at high temperatures (Bozinovic et al., 2013), increase the upper temperature limit (Jurriaans and Hoogenboom, 2020), performance breadth (Jurriaans and Hoogenboom, 2020) or heat resistance, like in tropical *Heliconius* butterflies (Nymphalidae) (Montejo-Kovacevich et al.,

2019). Therefore, studies have now highlighted the role of plasticity in shaping phenotypic responses to contemporary disturbances such as climate change (Charmantier et al., 2008).

Phenotypic plasticity can be a source of evolution if the resulting trait change goes in the direction favored by directional selection in the new environment. However, it would hinder evolution and maintain genetic variance if it enables to reach the new optimum (Ghalambor et al., 2007; Gibert et al., 2019). Yet, not all changes resulting from plasticity are adaptive. The direction of the change can be counter-beneficial to fitness-related traits. Warm development conditions in ectotherms are frequently associated to shorter development time, earlier sexual maturity, but smaller size and a lower reproductive success (Kingsolver and Huey, 2008; Merilä and Hendry, 2014).

3.3. Morphological variations along climatic gradients

In addition to physiology, morphology is one of the strongest drivers of thermal performance in ectotherms. Given the thermal advantage offered by darker body colorations, which allow light radiation to be absorbed more quickly and which results in faster heating, darker morphs should be favored and be the dominant phenotypes in colder climates, i.e. higher altitudes and latitudes. This assumption, also known as the thermal melanism hypothesis (TMH) (Clusella Trullas et al., 2007), has been extensively studied in numerous insect clades and at various geographical, continental and regional scales (Hegna et al., 2013; Zeuss et al., 2014; Xing et al., 2016, 2018; Stelbrink et al., 2019), showing the existence of such melanism coloration gradients along elevation and latitude (Fig 4). It thus reveals a genuine selective advantage of melanization in cold climates, as shown for instance in alpine butterflies *Colias* sp., in which melanistic individuals are less predated at high altitude because they are more efficient flyers (Ellers and Boggs, 2004; Roland, 2006). On the contrary, environments with higher insulation host species or individuals that harbor a lighter coloration, thereby avoiding overheating (Zeuss et al., 2014; Stuart-Fox et al., 2017). However, even if the number of studies, particularly on altitudinal gradients, is scarcer than in temperate regions, there is a mixed support for the thermal melanism hypothesis in the tropics. Species and assemblages can show divergent trends, sometimes running counter to TMH predictions (Dufour et al., 2018; Fiedler and Brehm, 2021), while other supporting it (Bishop et al., 2016; Xing et al., 2018).

As stated previously, visible colors represent only a small part of the incident solar radiation, and optical properties of a body can be disconnected to a certain extent between NIR and UV-visible (Stuart-Fox et al., 2017). Most recent studies have found that climatic conditions are reliable predictors of wing coloration in butterfly assemblages across Europe and Australia. Their most striking finding is that temperature is a better predictor of the reflectance in the NIR range than the UV-visible reflectance (Munro et al., 2019; Kang et al., 2021). The thermal melanism hypothesis suffers from an imbalance of studies on the subject between the tropics and temperate climates. Moreover, the study of optical properties beyond

UV-visible wavelengths, and their implications for heat absorption properties, in relation to climatic conditions remains in its early stages.

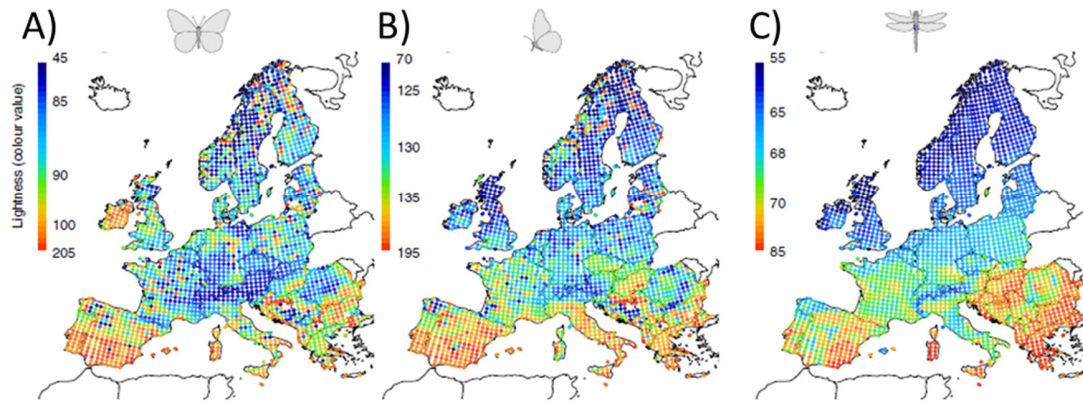


Figure 4. An illustration of the geographical gradient of body coloration in insect assemblages in Europe (Zeuss et al., 2014). The average color lightness of A) dorsal butterfly wings (434 species), B) ventral butterfly wings and C) dragonfly bodies (107 species), increases with decreasing latitude, in warmer climates, in other words, these insects are darker at higher latitudes. The authors hypothesize that thermal adaptation is the main driver of the observed trend. The color lightness values were calculated from photographs, each pixel was attributed a score between 0 (complete black) and 255 (complete white) and these values were averaged across all pixels to obtain a single-color value for each species.

A biogeographical rule, known as Gloger's rule, predicts a positive association between melanization in hotter and more humid environments. Mainly applied in endotherms, such observations have been made in ectotherms (Delhey et al., 2019). Melanin takes part in resistance against pathogens: when a pathogen penetrates the host, melanin is produced and used to encapsulate the foreign particle, thereby isolating it from the host. It also activates the production of oxygen radicals that are toxic to both the host and the pathogen, resulting in an efficient but costly defence for the host (Freitag et al 2005). Dark coloring is a way of evacuating excess melanin (True, 2003). A possible underlying mechanism would be the increased abundance of parasites and fungus in humid environments that would select for an increased production of melanin, involved in immunity (Nappi and Christensen, 2005), or inversely, a selection for darker individuals in colder climates which may result in an increased acquisition of melanin precursors, and in a bias towards melanin-based immune responses. Despite a support at an intra-species level, there is no evidence for a clear-cut correlation, when studied in Australian and European butterfly assemblages (Dalrymple et al., 2018; Kang et al., 2021). These biogeographical rules potentially have a simultaneous effect on the colors and patterns observed along climatic gradients. Therefore, adaptive variation in animal

coloration, especially in the UV-visible range, can be the result of a compromise between thermoregulation and multiple functions such as camouflage, intra and inter specific signaling, immunity, desiccation resistance, and protection against dangerous UV radiation (True, 2003; Körner, 2007; Clusella-Trullas and Nielsen, 2020). These selection pressures overlap and sometimes contradict one another (Lindstedt et al., 2009; Hegna et al., 2013), resulting in contrasting pattern of color variation, whose adaptive function is difficult to decipher.

Many studies have shown that wing coloration can be plastic in response to temperature and that such temperature-dependent plasticity has adaptive potential. It has been observed in many insect taxa, and studies that show an association between warm developmental temperatures and the decrease in darkness in adult coloration are numerous : between phenologically distinct populations of butterflies larvae (Hazel, 2002), following a climatic change in beetles (Zvereva et al., 2019), or as a result of experimental conditions, as in *Aglais urticae* (Nymphalidae) showing a direct link between degree of darkness and body heat absorption (Markl et al., 2022).

Thermal melanism is likely subject to the influence of individual size. Color has a greater thermal effect in larger ectotherms, due to body thermal inertia. This may explain why seasonal color changes are more prevalent in large reptiles (Norris, 1967), and color does not seem to be linked to thermal adaptation in small butterflies (De Keyser et al., 2015). In Colombia, both coloration and size are drivers of thermal tolerance in butterfly community (Khazan et al., 2022). There is some debate as to whether the size of ectotherms is in itself an adaptive trait in response to temperature. For a given volume, a shape with a larger surface area dissipates heat more rapidly, while large bodies retain heat longer. Bergmann's rule (James, 1970), states that an individual or species of colder climate will be larger than those living in warmer conditions. Originally described in endotherms, a review of the literature by Shelomi (2012) shows that in insects, such an association is not obvious, with as many studies supporting it as finding the opposite variation or finding no trend at all, whether along latitudinal or altitudinal gradient, and at intra or interspecific scale.

Finally, the significance of body coloration is modulated by behavioral thermoregulation. Although cold ambient temperature can be detrimental to ectotherms, heating through solar radiation makes it possible for them to colonize biotopes such as high altitude or high latitudes, via behavioral adjustment, especially basking (Norris and Kunz, 2012; Barton et al., 2014). Thus, high-altitude toads are able to increase their body temperature by more than 20°C via basking on sunny days (Lambrinos and Kleier, 2003), and life at high elevations is associated with more time spent basking during the day in vertebrate ectotherms (Hertz and Huey, 1981; Lambrinos and Kleier, 2003). Plus, thermal stresses have the potential to be reduced by microclimatic refuges. In the Andes, understory temperatures are lower than in the canopy, and this difference is more marked at low altitude (Montejo-Kovacevich et al., 2020), which could help animals to withstand periods of heat stress.

4. The puzzle of thermoregulation in clearwing butterflies

4.1. Evolution of transparency in butterflies

Lepidoptera, which includes moth and butterflies, are known to exhibit a great variety of colors and patterns on their large wings. Coloration may be pigmentary or structural in origin. The scale-covered wings contain pigments of various types such as melanins, (responsible for the dark colors) pterins, flavonoids, ommochromes, bile pigments or papiliochromes specific to Papilionoidea (Nijhout, 1985). Microstructures on the surface of the scales also interact with light resulting in very bright iridescent colors, as in the famous *Morpho* genus (Debat et al., 2018). The great variation in melanism observed arises from the many ecological implications of color in butterflies and their complex interaction, such as thermoregulation (Watt, 1968), aposematic signaling (Hegna et al., 2013), crypsis (Majerus, 2009), sexual selection (Kemp, 2007), masquerade (Skelhorn et al., 2010), disruptive coloration and predator deflection (Vallin et al., 2011), and pathogen resistance (Nappi and Christensen, 2005).

Species with transparent or translucent patches on their wings can be found in 31 families, which corresponds to approximately a quarter of the extant 124 Lepidoptera families (Gomez et al., 2021). Given that the vast majority of butterflies and moths are opaque and coloured, transparency has likely evolved several times independently in Lepidoptera. Transparency is the transmission of visible light through a material. Among the living organisms, transparency is mostly present in aquatic environment, in the pelagos, where it presents the advantage to offer camouflage in full sight (Johnsen, 2001). By contrast, in terrestrial organisms, transparency is rare. Insect wings are among the few examples of terrestrial transparency, along with there is the famous ‘glass’ frogs of the family Centrolenidae (Rudh and Qvarnström, 2013), the leaves of high-altitude Himalayan plants *Rheumobile* (Polygonaceae) (Omori et al., 2000) or the petals of the skeleton flower *Diphylleia grayi* (Berberidaceae), a species living in the mountains of Japan.

Three main physical reasons may explain why transparency is rare on land. First, the difference in refractive index is greater between chitin and air than between chitin and water, which increases the reflection of light thereby cancelling the transparent effect (Pomerantz et al., 2021). Second, terrestrial environment is more constraining for UV radiation that is filtered out in water. By absorbing UV, melanin provides protection against this deleterious radiation. Third, terrestrial life requires sufficient load bearing through relative tissue thickness and rigidity, because the buoyancy of air is lower than water. Organic tissues absorb visible radiation, and this absorption increases proportionally with thickness (Zaret, 1981).

In butterflies, transparency is achieved via a great diversity of structural modifications in shape, insertion angle, coloration, or density: scales absent or highly modified in shape (reduction in width, reducing them to piliform scales), a reduction of scale density on the wing membrane, scales in higher density than opaque scales but pigmentless, scales erect on the wing membrane letting light go through (Gomez et al., 2021) (Fig 5). The membrane itself of

transparent wings undergoes structural modifications at the nanoscopic scale. The nanostructures on the surface of the wings of some butterflies and moths limit the reflection of the light by forming a progressive increase of the refractive index on the surface of the tissue (Yoshida et al., 1997; Siddique et al., 2015; Pomerantz et al., 2021). In butterflies, a great diversity of nanostructures shapes results in a wide range of light transmission percentage through the membrane (Pinna et al., 2021).

Although it may seem anecdotal, the evolution of transparency in butterflies raises serious questions about evolution, ontogeny, and ecological implications, especially thermoregulation. At a multi taxon scale, a recent study on wing transparency in Lepidoptera has revealed that transparent patches tend to transmit less light in the visible and near infrared (400 to 1100 nm), and therefore absorb more, in high latitudes than in tropical climates (Gomez et al., 2021). Following that, one might expect transparency to decrease with altitude as well. These preliminary results suggest a thermal cost of transparency in butterflies, yet, it does not provide any clues as to the thermal properties and absorption of the wings at longer wavelengths.

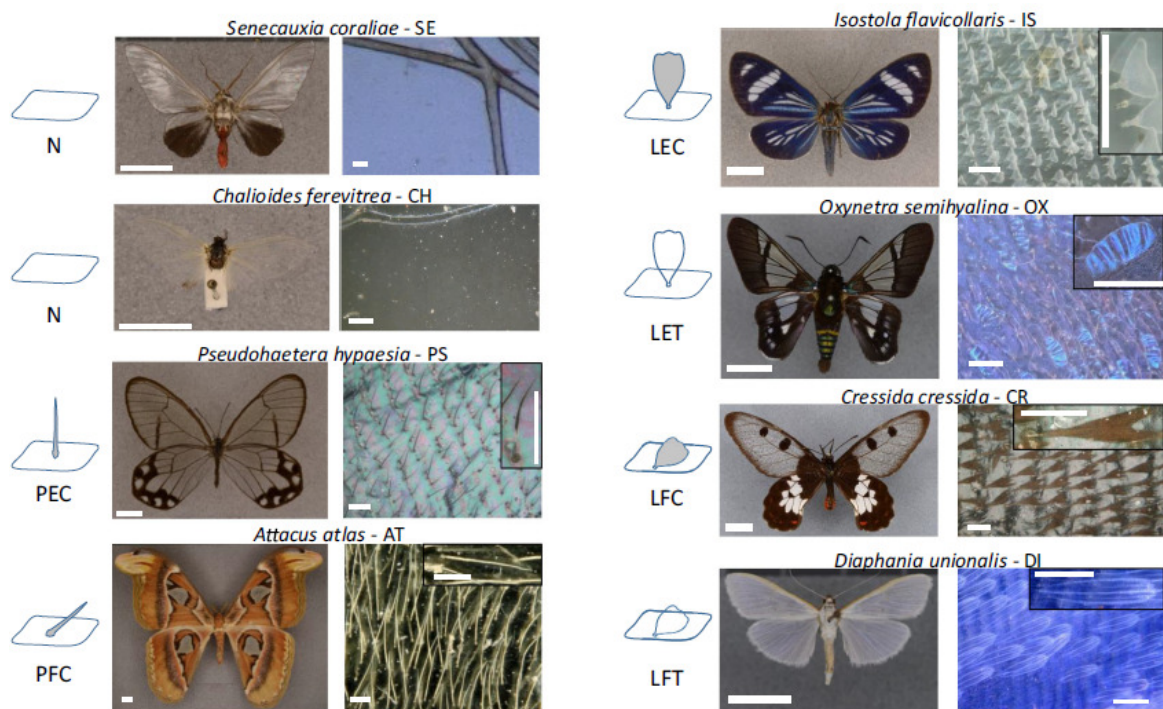


Figure 5. Examples of the diversity of scale morphologies to achieve transparent wing patches among Lepidoptera. The scales can be piliform (P) or lamellar (L), they can be erect (E) or flat (F) on the wing membrane, and can contain colored pigments (C) or they can be transparent (T). In some species the wing membrane does not bear scales (N). after Gomez et al (2021).

4.2. Ithomiini butterflies as a model for studying thermoregulation

Among Lepidoptera, the Neotropical tribe Ithomiini (Nymphalidae) represents an outstanding group of butterflies. Almost 80% of its 396 species have transparent or partially transparent patches on their wings (Fig 6,7). These butterflies, found from Southern Mexico to Northern Argentina, have started diversifying 26.4 million years ago (Chazot et al., 2019) in the Andean region, which constitutes the main hotspot for this clade in terms of species richness (Doré et al., 2022) (Fig 8). This high richness in the Andean region, and more generally the dynamic of Ithomiine evolution, is closely linked to regional historical geology: around 23 million years ago, the Amazon region experienced a marine incursion, from Peru to Colombia, known as the Pebas formation, resulting in a loss of habitat and the extinction of most Amazonian lineages (Chazot et al., 2019). During this time, there was no sign of migration between northern and southern Andean populations, separated by the waters. It was only 10 million years ago that mountain growth finally pushed the water towards the ocean, and marked a great diversification, dispersal and exchange between the Andes and Amazonia. The other parts of the Amazon and peripheral regions were the last to be colonized (Chazot et al., 2019). Today, Ithomiine species are highly segregated along the elevation gradient in the Andes, and some species can be found up to 3000 meters, a remarkably high altitude for diurnal tropical butterflies (Chazot et al., 2014).

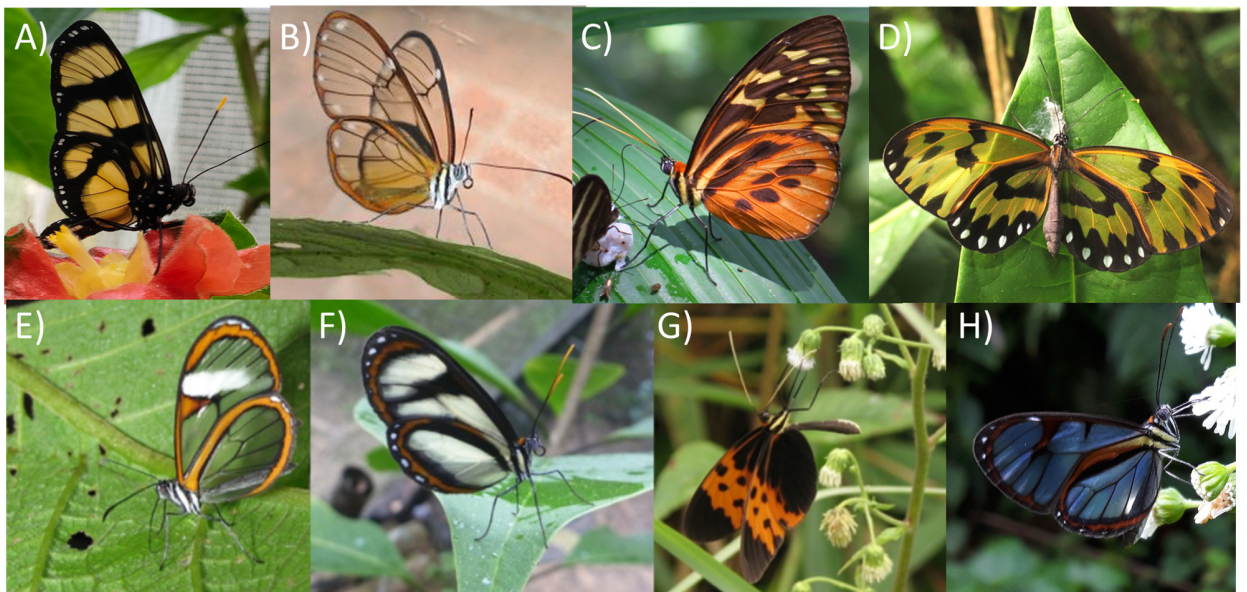


Figure 6. Examples of wing color pattern diversity among Ithomiine butterflies. Species :
A) *Methona confusa* (photo: Violaine Ossola), B) *Hypomenitis enigma* (photo: Marianne Elias), C) *Tithorea harmonia* (photo: David Geale), D) *Veladyris pardalis* (photo: Anderw Neild), E) *Pteronymia alissa* (photo: Marianne Elias), F) *Ithomia salapia* (photo: Mélanie McClure), G) *Melinaea mothone* (photo: Marianne Elias), H) *Ithomia diasia* (photo: Marianne Elias).

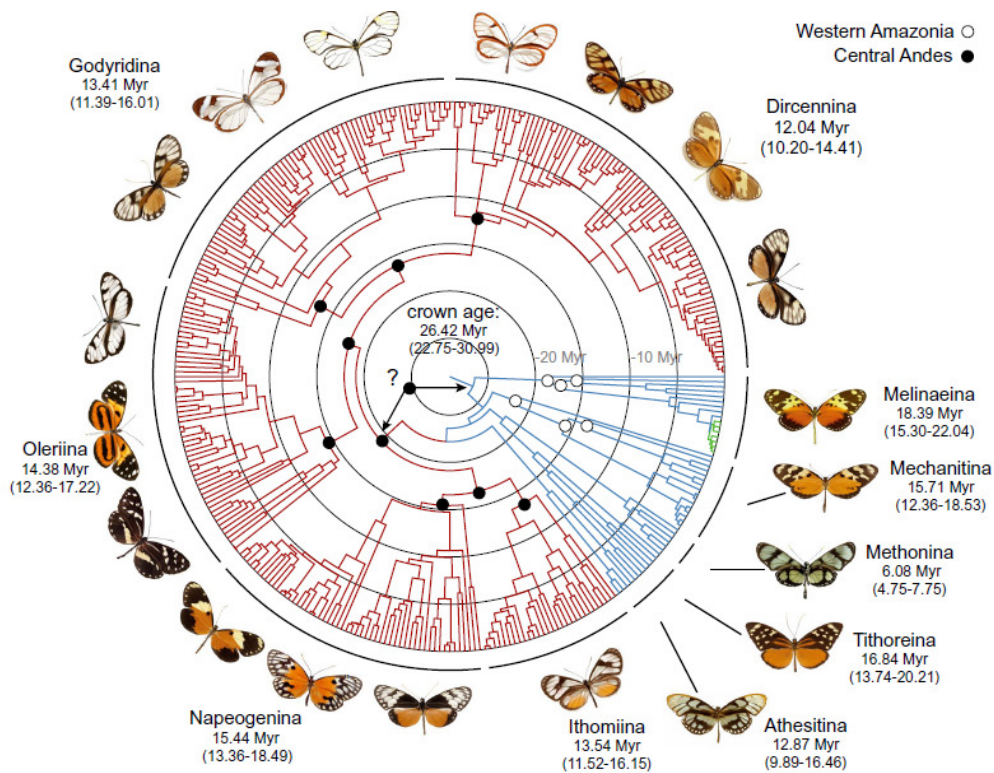


Figure 7. Chazot et al., 2019's Molecular phylogeny of 339 Ithomiini species out of the 396 recorded, based on mitochondrial and nuclear gene fragments. The tree topology was obtained under maximum likelihood inference, and branch lengths were estimated by Bayesian inference. This tree gives an idea of how wing pattern and phylogeny are dissociated in Ithomiini.

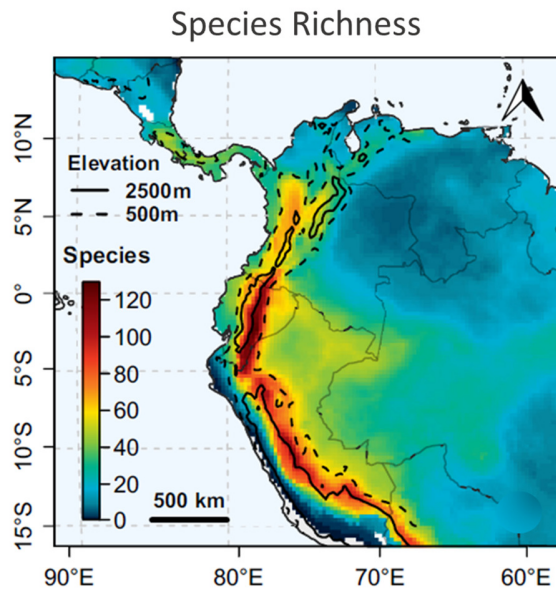


Figure 8. This map of the north-western region of the Neotropics, from (Doré et al., 2022) shows the geographical distribution of the species richness of Ithomiini butterflies. It clearly demonstrates the biodiversity-rich nature of the Andean region, which is a hotspot for these butterflies.

Recently, Ithomiine butterflies have been the subject of various ecological studies with standout results. The widespread presence of transparency on their wings is surprising, especially because these butterflies have the particularity to be toxic, via the presence of pyrrolizidine alkaloids, and aposematic (i. e., their wing patterns are warning colorations that predators learn to avoid), and as such Ithomiine butterflies are part of mimicry rings, where co-occurring species have converged in warning color patterns (Beccaloni, 1997). Surprisingly, unpalatability tends to be stronger in clearwing than in opaque species (McClure et al., 2019). Pinna et al. (2021) suggest that transparent patches are in fact involved in the aposematic signal. On the other hand, it has been shown that transparency reduces detection and attack by naïve predators (Arias et al., 2019, 2020, 2021). Transparency is therefore involved both in crypsis and in aposematism. The study of the altitudinal distribution of mimicry rings gives us an indication of the variation in wing pattern along elevation in the Andes. It seems that opaque butterflies are restricted to low altitudes, while mimicry rings involving clearwing species are existing up to very high altitudes (Chazot et al., 2014) (Fig 9), a surprising result in the light of what we know about thermal melanism. The absorption of near infrared (NIR), which, unlike lower wavelengths, are not visible to animals and not subject to biotic selection pressures, could constitute a compensation mechanism for heating accumulation, especially in high altitude species.

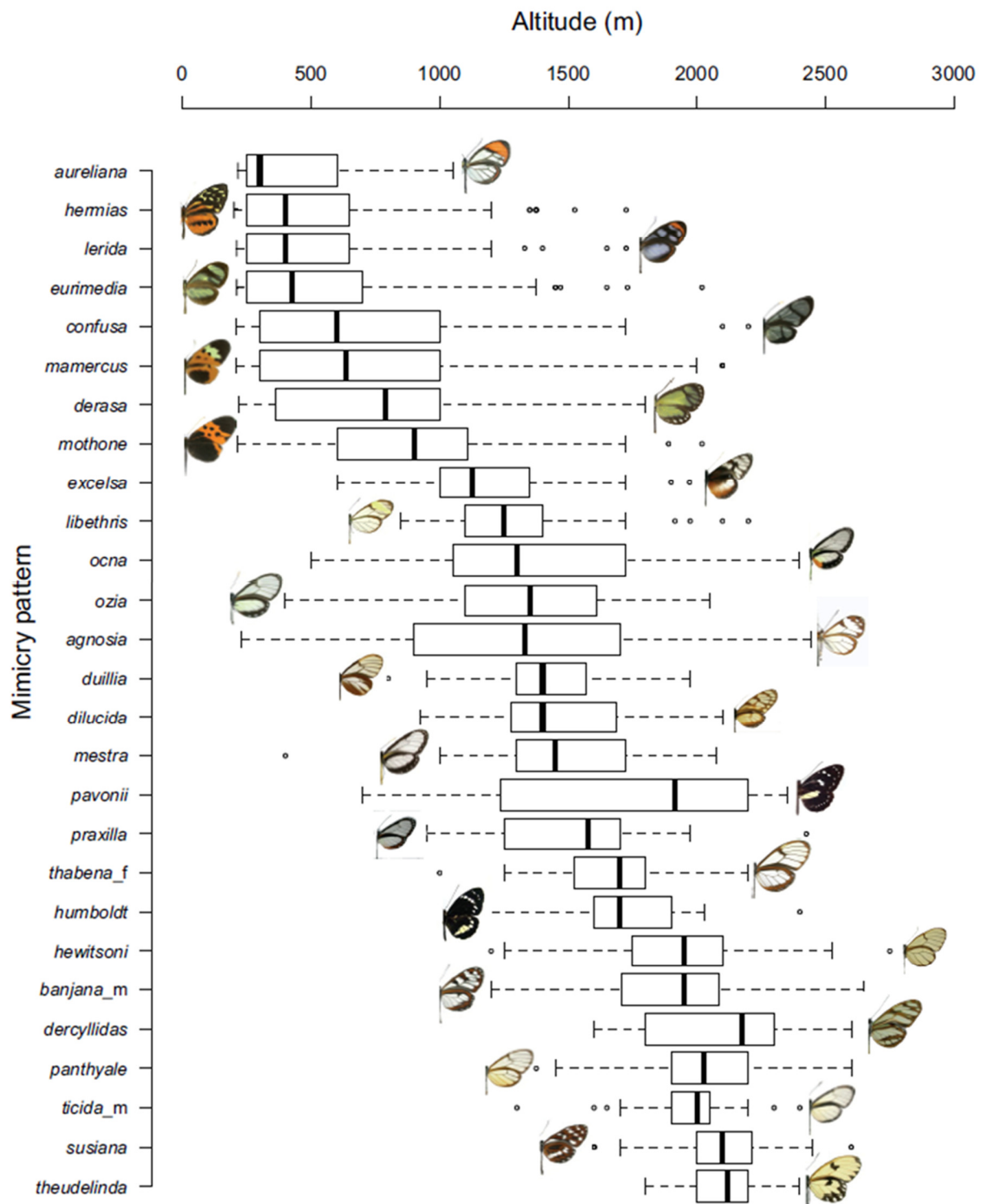


Figure 9. Chazot et al (2014) showed that Ithomiini mimicry rings that include opaque species are only found in low to mid altitudes, in the Andean mountains, while transparent mimicry rings are found at all elevations, and as high as 3000 meters above sea level.

5. Aim of the thesis project and main questions

Past studies on thermal adaptation undeniably show the importance of a multidisciplinary approach in ecology. Ithomiini butterflies represent an original system that challenges our knowledge of ecological concepts because they feature an exceptional characteristic, wing transparency. Their high diversity, with repeated colonization events of habitats at different altitude, provides strong statistical power to investigate physical and physiological responses to climatic conditions along a tropical altitudinal gradient.

In this thesis we will address the main following questions

How are thermal properties of transparent wings linked with climatic conditions? In the first chapter, we will explore the thermal consequences of transparency and the distribution of wing properties along the elevation gradient. We will compare the optical and thermal properties of the wings of 42 ithomiine species of varying opacity, at different wavelengths, and assess whether there is a thermal cost to transparency, and whether it is compensated for by near-infrared absorption. We will examine how these properties are distributed along a climatic gradient, and test if the biogeographical rules of thermal melanism apply to these clearwing butterflies.

Is there a physiological adaptation to temperature along an altitudinal gradient? In the second chapter, we will focus on the metabolic adaptation of these butterflies to altitude, by comparing two communities at high and low altitudes in the Ecuadorian Andes. We will compare their thermal requirements *in natura*, while noting the potential thermoregulatory behavior of different species in the field. In the laboratory, we will evaluate the thermal tolerances of species from these communities to cold and hot stress, and determine whether any differences of tolerances may be due to adaptation or phylogenetic conservatism.

Are physical and physiological thermal responses plastic? In the third chapter, we will assess the thermally-induced plasticity of traits linked to thermal adaptation of these butterflies in two species of Ithomiini from Panama, by rearing them at different temperature treatments. We will examine larvae survival and development time, as well as potential changes in adult coloration and adult tolerance to heat and cold. It will highlight the potential sensitivity of immature stages to temperature and the potential room for adaptation of these butterflies, especially in the current context of climate change.

Overall, answering these questions regarding thermoregulation in Ithomiini, and especially in clearwing species, will help understand at which level these butterflies play to buffer the variations in temperature of their environment, whether intrinsic physical properties emerging from the butterfly's structure are primordial, if behavioral adjustments help to compensate for intrinsic limitations, and to what extent the plasticity of these traits contribute to the evolution of this lineage of tropical butterflies.

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Chapter 1. Clearwing butterflies challenge the thermal melanism hypothesis

Submitted for publication

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Abstract

In contrast to most butterflies harboring opaque wing colorations, some species display large transparent patches on their wings. Wing transparency, which entails a dramatic reduction of pigmentation, raises the question of potential costs for vital functions, such as thermoregulation, especially along climatic gradients. The thermal melanism hypothesis posits that darker colorations should be favored in colder environments, which enables them to absorb more radiation and maintain a body temperature compatible with activity. This prediction extends to the near infrared (NIR) range, which represents a large proportion of solar radiation. Here we assess the implications of wing transparency for light absorption and thermal properties in 42 butterfly species from the neotropical tribe Ithomiini that range the extent of transparency, from fully opaque to highly transparent, and we test whether those species conform to the prediction of the thermal melanism hypothesis. We find that transparent wings are less efficient than opaque wings to absorb light across UV, Visible and NIR wavelength ranges, and are also less efficient to collect heat. Moreover, dark coloration occupies a lower proportion of wing area as altitude increases, and ithomiine species harbor more transparency at higher altitudes, where climatic conditions are colder, going strongly against the prediction of the thermal melanism hypothesis. We discuss these surprising results in light of recent studies suggesting that factors other than adaptation to cold, such as predation pressure, physiology or behavior, may have driven the evolution of wing patterns in Ithomiini.

1. Introduction

Diurnal butterflies are known for the great variety of colors they display. The scales covering their wings produce colorations through pigments (Stavenga et al., 2014) or structures (Tada et al., 1997; Berthier, 2007). Coloration is involved in multiple functions, including intraspecific signaling (Kemp, 2007); antipredator defenses such as crypsis and camouflage (Endler, 1984), masquerade (Skelhorn et al., 2010), aposematism and mimicry (Poulton, 1887); as well as thermal adaptation (Wasserthal, 1975). The balance between these selective pressures drives the diversity of color patterns in butterfly wings (Postema et al., 2022).

In ectotherms, coloration has been shown to play an important role in determining body temperature and activity, since the degree of darkness is positively correlated with internal body temperature (Heinrich, 1973). Darker butterfly wings absorb more light, and are associated with longer flight activity and larger flight distances in cold conditions than lighter wings (Roland, 1982; Ellers and Boggs, 2004).

The thermal melanism hypothesis (TMH) posits that darker colorations provide thermal advantages in cold environments (Clusella Trullas et al., 2007), especially for ectotherms, as dark surfaces are more efficient to absorb heat from the environment than light surfaces. Darker species or individuals are expected to live at high altitudes or latitudes, whereas lighter ones are more likely to be present at lower altitudes and latitudes, which offer warmer conditions. The existence of such melanism gradients has been shown in many case studies, across geographical regions and wide ranges of taxa, from reptiles to insects (Guppy, 1986; Clusella-Trullas et al., 2008; Zeuss et al., 2014). However, visible colors represent only a small part of the incident solar radiation reaching the earth's surface. Over 50% of the solar energy lies in the near-infrared wavelengths (NIR, 700 to 2500 nm). NIR should then be of particular importance in ectotherm thermal capacities, more than ultraviolet to red (i. e. 300 to 700 nm, hereafter UV-Visible range) (Stuart-Fox et al., 2017), since they are not involved in other aspects of their ecology because they cannot be seen by most organisms. Recent studies on communities of European (Kang et al., 2021) and Australian (Munro et al., 2019) butterflies confirmed that reflectance in the NIR range is strongly correlated with climatic factors, especially temperature, and more strongly compared to UV and Visible wavelengths. In colder environments, butterflies tend to reflect less and therefore absorb more in the NIR. These findings highlight that optical properties can be disconnected to a certain extent between NIR and UV-Visible ranges. By contrast, no clear trends exist regarding humidity or precipitation and wing darkness. Gloger's rule, which predicts that darker individual should be found in more humid climates, has been evidenced mainly for endotherms with little clear support in ectotherms (Delhey et al., 2019; Kang et al., 2021).

In Lepidoptera, few species exhibit transparency on their wings, a feature that has evolved multiple times independently (Gomez et al., 2021). Transparency is achieved by a large diversity of wing microstructures, including a combination of scale shape or insertion modification, reduced scale size or density, as well as a reduction of scale pigments (Gomez et

al., 2021). In many cases, transparency is enhanced by the presence of nanostructures on the wing membrane, which decreases reflection (Siddique et al., 2015; Pinna et al., 2021; Pomerantz et al., 2021). Transparency has attracted recent research effort regarding its now-established role in crypsis in various species (Arias et al., 2019, 2020, 2021), and its involvement in predator-prey signaling in some clades of mimetic Lepidoptera (Pinna et al., 2021). Yet, transparency may come at the cost of other functions involving wing coloration, notably thermoregulation. While thermoregulatory processes are well documented in opaque species, they are largely unknown in clearwing butterflies. A study focusing on wing transparency across the entire Lepidoptera order found that clearwing species tend to transmit less light, and thus absorb more, in high latitudes than in tropical climates, suggesting a thermal cost for butterflies (Gomez et al., 2021). Yet, the actual impact of transparent wing patches on wing thermal properties needs to be investigated.

Among Lepidoptera, Ithomiini is a remarkable neotropical tribe of ca. 400 species (Chazot et al., 2019; Doré et al., 2022), 80% of which exhibit transparency on their wings to some extent. Species are highly segregated along the elevational gradient, from warm lowlands up to colder highlands, as high as 3,000 meters (Chazot et al., 2014; Doré et al., 2022). Because wing transparency in Ithomiini is achieved by a reduction in membrane coverage by scales (Gomez et al., 2021; Pinna et al., 2021), and is therefore associated with a lower quantity of pigments, one might expect those areas to poorly absorb solar light. Previous studies of geographical distribution of Ithomiini wing patterns revealed that the proportion of clearwing species increases with altitude, to the extent that all species above 2000 m have transparent wings (Chazot et al., 2014; Doré et al., 2022), contrary to the predictions of the TMH. Yet, this observation is based on the UV-Visible range (Chazot et al., 2014; Pinna et al., 2021; Doré et al., 2022), and optical transparency shown in that range may be dissociated from the absorption properties in the NIR.

Here, we investigate for the first time wing traits relevant for thermal adaptation in 42 transparent and opaque Ithomiini species that represent the diversity of wing colorations, by using Infrared imaging and spectrophotometry. We assess the distribution of these wing traits in relation to the climatic conditions the species are exposed to in a phylogenetic framework. Specifically, we characterize and quantify the optical and thermal properties of transparent and opaque wing patches to investigate two mutually exclusive scenarios:

(i) *Absorption compensation*: in this scenario, the higher transmission and lower absorption of wavelengths in the UV-Visible range of transparent wing areas are compensated by a high absorption in the NIR, and the thermal capacities of transparent wings may be similar to or even better than those of opaque wings. Under this hypothesis, adaptation to climatic conditions may drive the altitudinal distribution of wing patterns in Ithomiini.

(ii) *Thermal cost*: in this scenario, the disconnection between wavelength ranges is not significant enough, with transparent wings transmitting more and absorbing less in the NIR range than opaque wings, therefore, reflecting similar absorption patterns at shorter wavelengths. This results in transparency being costly in terms of intrinsic heating capacities. Under the thermal

cost scenario, thermal properties may not be the main target of selection with regard to the altitudinal distribution of wing patterns in Ithomiini.

In addition, assuming that opaque patches - even if not covering a high proportion of the wing surface in transparent species – may follow the thermal melanism hypothesis, we tested whether they represent a higher proportion of wing surface in species found in colder climates, a mechanism that could also compensate for poorer thermal capacities of transparency under the *thermal cost* scenario. The absence of evidence of compensation mechanisms (disconnection NIR from UV-Visible range, or increased proportion of opaque patches with altitude) would imply that factors other than wing color patterns, such as behavior or metabolism, explain adaptation to colder highland habitats in Ithomiini.

2. Results

To assess the thermal adaptation of Ithomiini butterflies, we examined the thermal and optical properties of the wings of 42 species in relation to climatic conditions. Unless specified otherwise, all statistical tests implemented Bayesian mixed models with Markov Chain Monte Carlo correcting for phylogenetic relatedness, with an associated Bayesian posterior P-value set to 0.05.

Wing optical and thermal properties

We first tested whether optical properties in the NIR wavelengths were correlated with those properties in the UV-Visible range, and whether those properties differed between transparent and opaque species, as defined hereafter. We measured transmission and reflection spectra on a 5mm-diameter spot on one hindwing and one forewing for each specimen, spanning the UV-Visible (300-700 nm) and NIR (700-2500 nm). We used the physical principle stating that $\text{Transmission}(\lambda) + \text{Reflection}(\lambda) + \text{Absorption}(\lambda) = 1$ to compute the total absorption spectra over 300-2500 nm (Fig. 4C). The transmission of the patch in the UV-Visible range was set as a measure for the degree of optical transparency. We split the species in two categories: transparent, for which wing transmission was higher than 35%, and opaque for the others (Table S1a, S1b, Fig.S1).

Overall, UV-Visible transmission (300-700 nm), i.e., transparency as perceivable by butterflies and predators, was a good predictor of both overall absorption (300-2500 nm) and NIR absorption (700-2500 nm, Table S2, Fig. 1). NIR and UV-Visible absorption were highly correlated (Table S2, Fig. 1), be the species transparent or opaque. More transparent patches absorbed less light than opaque patches whatever the wavelength considered, supporting the *thermal cost* scenario for transparency (Fig. S2).

To investigate the thermal capacities of transparent wings and test whether they differ from those of opaque wings, we heated up wings with a flash illumination, and imaged them with an infrared camera to record temperature across the wing and its variation through time (Fig. 4A-B). Image analysis showed that the average heating capacity of the wing spot, represented by excess temperature ΔT (maximum temperature reached after illumination - initial temperature), was closely related to its transparency, as estimated by the transmission in the UV-Visible range (Table S3, Fig. 2). Hence, the more transparent the spot, the less it heated up, in agreement with the *thermal cost* scenario. ΔT of wing spots was also positively predicted by the mean total absorption of the spot and by its NIR absorption (Table S3, Fig. 2).

Therefore, wings that absorbed more in the UV-Visible-NIR range or in the NIR range heated up more. In an in-depth exploration of the intra-wing variation in heating capacity, we extracted ΔT on various points on different parts of the wings (veins and cells, i.e., the parts between veins), either in darkly colored patches DP (black to brown colors), lightly colored patches LP (for opaque species only, yellow to orange colors), or transparent patches TP, and

on light LV or dark veins DV. A close look at the different areas and structures of the wings revealed that dark patches heated up significantly more than any other coloration on the wings, transparent or light (DP>(LP,TP) in Table S4, Fig. S3). Moreover, light patches heated up significantly more than transparent patches (LP>TP in Table S5, Fig. S3). Dark patches heated up more than dark veins (DP>DV in Table S6, Fig. S3), but on the other hand, light veins absorbed more heat than light patches (LV>LP in Table S7, Fig. S3). In all cases, the position of the patch with respect to the thorax, proximal or distal, played a role in predicting ΔT , but this varied according to the color of the patch considered, showing no clear-cut trend (Table S4-7).

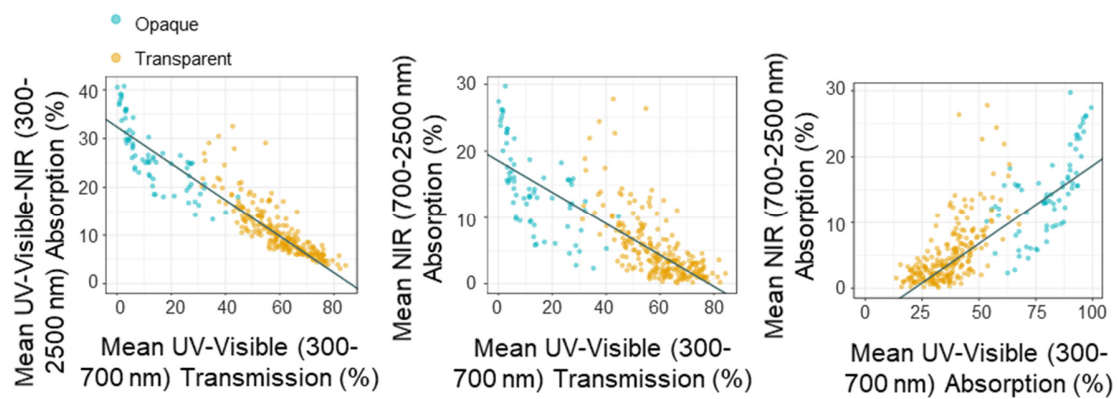


Figure 1. Relationship between absorption and transmission spectra at different ranges: overall UV-Visible-NIR (300-2500 nm) absorption against UV-Visible (300-700 nm) transmission, NIR (700-2500 nm) absorption against UV-Visible transmission, NIR (700-2500 nm) absorption against UV-Visible (300-700 nm) absorption. All relationships are significant and the solid line is drawn from the prediction of the Bayesian phylogenetic mixed model (see Table S2) (N opaque = 72, N transparent = 235).

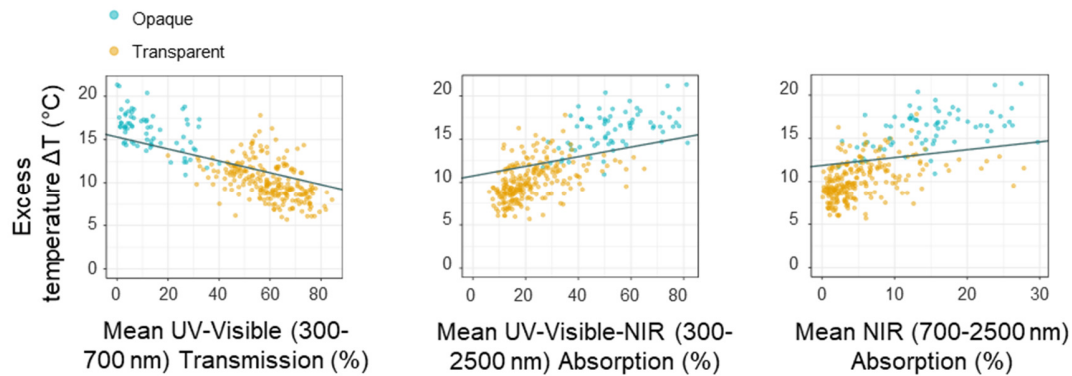


Figure 2. The thermal properties of wing spots represented by the Excess Temperature ΔT ($T^{\circ}\text{final} - T^{\circ}\text{initial}$) under a flash illumination, extracted from the thermal imaging, against their optical properties at different ranges: UV-Visible (300-700 nm) transmission, UV-Visible-NIR (300-2500 nm) absorption, and NIR (700-2500 nm) absorption. All relationships are significant and the solid line is drawn from the prediction of the Bayesian phylogenetic mixed model (see Table S3) (N opaque = 72, N transparent = 235).

Wing trait distribution along climatic gradients

We tested whether climatic variables predicted the proportion of dark patches on the wings of all the species, as posited by the TMH. We extracted 19 published average climatic variables for each species (Doré et al., 2022) that included temperature and precipitation descriptors and performed a PCA. PC1 and PC2 explained respectively 49 % and 27.1 % of the total variation. Higher PC1 values corresponded to warmer climates, while higher PC2 values corresponded mainly to wetter and more time-stable climates (tables S8, S9, Fig. S4). Pearson's product-moment correlation tests showed that PC1 was strongly negatively correlated with altitude ($R=-0.95$, $N=42$, $p < .0001$), meaning that species inhabiting higher altitude habitats had a lower PC1 value. PC2 did not correlate with altitude ($R=0.24$, $N=42$, $p=0.12$).

To test if opaque and transparent species had similar climatic ranges we used the Levene's heteroscedasticity test on climatic variables. We found that opaque species were restricted to warmer environments, while transparent species were ubiquitous and not associated to particular altitudes ($F = 110.71$, $p < .005$) (transparent species: mean=-0.27, var=10.56, opaque species: mean=1.08, var=1.09), in contradiction with the TMH. Opaque and transparent species did not significantly differ in their variance for PC2 ($F=1.76$, $p=0.19$) (transparent species: mean=0.29, var= 3.88, opaque species: mean=-1.89, var=3.45), showing that they did not segregate according to humidity or environmental stability gradients. Overall, climate did not predict wing optical properties. The degree of transmission of the wing spots over the range UV-Visible were not predicted by PC1, with a negligible positive effect for PC2 which goes against Gloger's rule (Table S10, Fig. S5). There was no significant association of spot absorption in the UV-Visible-NIR (Table S11) or NIR ranges (Table S12) with PC1 or PC2. By contrast, the average ΔT of the whole wing surfaces was positively correlated to PC1

(Table S13, Fig. 3). In other words, species living in warm environments heated up more than butterflies living in colder environments.

Across all species, the proportion of dark surface on the wings increased with PC1 and was therefore higher in warmer environments (Table S14, Fig. S6). We found a similar trend when restricting the analysis to only transparent species (Table S15, Fig. S7). We examined whether this proportion varied with climate within the proximal zones alone, because wing conduction could be limited, and a patch was expected to have a greater thermal effect when closer to the body. Again, the proportion of dark surface in the proximal zones increased with PC1 (Table S16, Fig. S8), and was therefore higher in warmer environments, in contradiction with the TMH. The proportion of dark surface did not correlate with PC2, except when considering only proximal zones, indicating that proximally darker wings were associated with more humid habitats. Finally, we found no effect of climatic variables on total wing area, a proxy for butterfly size (Table S17).

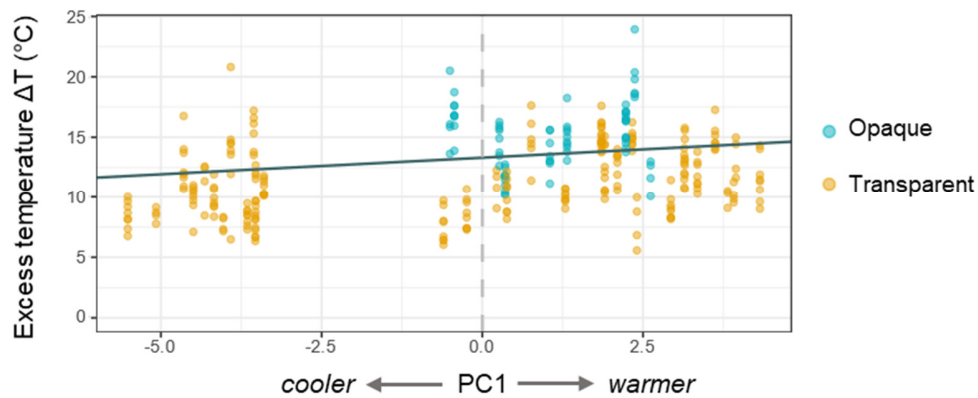


Figure 3. Wings mean Excess Temperature ΔT ($T^{\circ}\text{final} - T^{\circ}\text{initial}$) under a flash illumination, extracted from the thermal imaging, as a function of climatic principal component PC1. The relationship is significant and the solid line is drawn from the prediction of the Bayesian phylogenetic mixed model (see Table S13) (N opaque = 72, N transparent = 235).

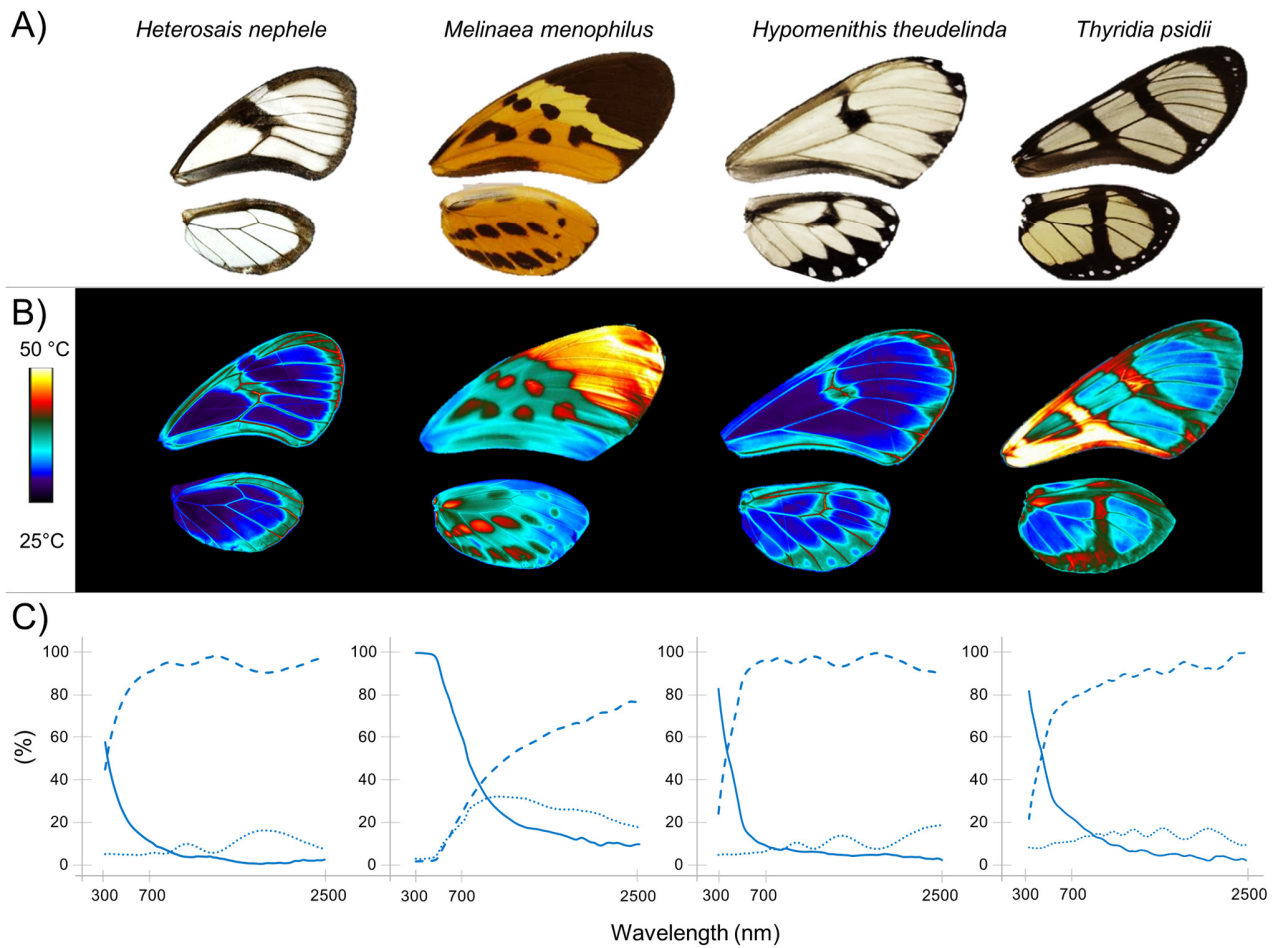


Figure 4. Examples of optical and thermal imaging on the wings of four species of different opacity. A: Photos under standard illumination of the hindwings and forewings of four of the species studied, *Heterosais nephele*, *Melinaea menophilus*, *Hypomenithis theudelinda*, *Thyridia psidii*. B: Visualization of the thermal imaging in false colors of the same wings under a flash light. C: Corresponding measured transmission (dashed lines) and reflection (dotted lines) spectra in percentage, for a spot of 5 mm of diameter on the proximal forewing of each butterfly, from 300 to 2500 nm, and the resulting computed absorption spectra (solid lines) for each wavelength λ as $\text{Absorption}(\lambda) + \text{Transmission}(\lambda) + \text{Reflection}(\lambda) = 1$.

3. Discussion

Thermal properties correlate with optical properties in clearwing butterflies

The thermal imaging of Ithomiini wings reveals that opaque wings are on average more efficient to heat up than transparent wings. Hence, transparency entails thermal costs, a conclusion supported by the close-up study of the different wing patches, in agreement with the *thermal cost* scenario. The darker the color, the more the patch heats up. Therefore, transparent patches contribute little to wing heat absorption, in line with the long studied link between melanization and heating capacities (Schmitz, 1994). Consistent with this finding, the optical examination of the transparent wing spots reveals that the low absorption in the UV-Visible wavelength range is not compensated by a higher absorption in the invisible NIR range, and therefore wing transparency comes at the cost of light absorption. This result departs from findings in other organisms where visible reflectance tends to be a poor predictor of NIR reflectance (Nussear et al., 2000). Moreover, transparent spots always absorb less light than opaque ones, regardless of the wavelength considered. This is in agreement with the correlation of the UV-Visible and NIR spectra that has been previously described for transmission (Gomez et al., 2021) or reflection (Munro et al., 2019; Kang et al., 2021) in interspecific studies of butterflies taxa. Yet, veins in transparent Ithomiini species are always covered with melanized scales. Those veins heat up significantly more than transparent and even lightly colored patches, and likely represent a major part of the overall wing thermal capacities by providing heat to the dynamic hemolymph flow system to the thorax (Tsai et al., 2020). In Ithomiini, absorption decreases in long wavelengths, and is quite low in the NIR for all the studied species. An interspecific comparison of the absorption of longer wavelengths (~8-12 μm), especially infrared radiated by the environment, remains to be carried out as this wavelength range is supposed to have greater thermal impact. However, the thermal imaging results clearly show that transparent patches are poorer at accumulating heat than opaque patches, suggesting an absence of compensation in the far infrared range.

The altitudinal distribution of transparent species contradicts the thermal melanism hypothesis

This study confirms the counterintuitive altitudinal distribution of Ithomiini wing patterns (Chazot et al., 2014). While the opaque species are only found in warm climates, clearwing species are ubiquitous and widely distributed, from hot lowlands to the cold Andean highlands. Moreover, among clearwing species, the proportion of opaque patches on the wings decreases in colder environments, i.e. at higher altitudes, challenging the long history of studies supporting the TMH in butterflies and insects e.g. (Guppy, 1986; Roland, 2006; Hegna et al., 2013; Zeuss et al., 2014), but adding to the observation that TMH does not apply to all taxa or communities. For instance a recent study found support for TMH in geometrids but not in noctuids found in the same study sites (Heidrich et al., 2021). Yet, these studies often focus

on temperate climates. Neotropical case studies are scarce and show mixed support to the TMH. Two closely related neotropical butterfly genera, *Catastica* and *Leptophobia* have a divergent coloration distribution along altitudinal gradients (Dufour et al., 2018), possibly explained by different behaviors and elevation ranges. While body and wing colorations become darker with increasing altitude in *Catastica*, *Leptophobia* shows a reversed pattern, similar to that observed in *Ithomiini*. In the case of *Ithomiini*, not only the non-conformity to TMH is outstanding, but there is no high NIR absorbance to compensate the poor UV-Visible absorption that would make highland butterflies more adapted to cooler conditions.

The NIR range is expected to be only subjected to thermoregulatory pressures, unlike UV-Visible wavelengths involved in vision. Indeed, NIR reflection spectra of Australian and European butterflies communities are well predicted by climatic conditions (Munro et al., 2019; Kang et al., 2021). In contrast to their conclusions, our study of the absorption properties of wing spots showed that in general, climatic conditions do not predict wing absorption in any radiation range. Moreover, the exploration of wing heating capacities shows that *Ithomiini* species living in colder environments are less efficient to heat up than species living in warmer climates, adding even more to the thermal cost of transparency. Finally, there is hardly any link between thermal or optical properties and humidity, and as such this study does not support Gloger's rule in *Ithomiini*.

Adaptation to cooler conditions is not the only thermal constraint faced by organisms, as high temperature may also be challenging. Here we found that opaque species, which absorb more radiation and have higher thermal capacities, live in environments where they are most exposed to heat. For ectotherms, avoiding overheating (i.e. increasing body temperature above a critical temperature threshold) can be challenging. Butterflies may have physiological adaptations (Montejo-Kovacevich et al., 2020) or behavioral adjustments, such as changing the position of their wings according to environmental radiation (Tsai et al., 2020). Information on the metabolism and behavior of *Ithomiini* *in natura* would shed light on their altitudinal adaptation, but are currently lacking.

In addition, microhabitat variability can result in individuals sharing the same overall environment being exposed to contrasting light and temperature conditions. Microhabitat preferences thus likely play a role in adjusting body temperature (Kleckova et al., 2014; Montejo-Kovacevich et al., 2020, 20). However, in lowland *ithomiine* communities, butterflies with transparent wing patterns mostly fly in the understory, close to the vegetation and out of direct sunlight, while the proportion of species with opaque wings increases with height in the canopy (Willmott et al., 2017). The latter are therefore more exposed to solar radiation, another counterintuitive distribution in relationship to thermoregulation.

Transparency under multiple selection pressures

Colorations in butterflies, and therefore wing optical properties, are under multiple selection pressures as they are involved in anti-predator defenses, signaling to conspecifics and thermal adaptation, with potential trade-offs (Postema et al., 2022). Consequently, selection for crypsis and aposematism could be detrimental for thermal capacities (Heidrich et al., 2018). Wood tiger moths are a good example of the phenomenon: larvae with large black patches are at a thermal advantage compared to larvae with equally black and orange patches, but the latter display a more efficient aposematic signal (Lindstedt et al., 2009). Imagoes display latitudinal variation in wing pattern, consistent with the TMH, which incurs cost in the efficiency of warning signals as melanic individuals experience more predation (Hegna et al., 2013).

In Ithomiini, the existence and pervasiveness of transparency is surprising given that those butterflies are toxic and harbor aposematic signals. Previous work has shown that transparency in Ithomiini reduces detection and attacks by naïve predators (Arias et al., 2019). Even though transparent patches are likely part of the aposematic signal (Pinna et al., 2021), transparency may reduce predator learning speed and memorization of prey, as indirectly suggested by the observation that unpalatability tends to be stronger in clearwing than in opaque species (McClure et al., 2019). Thus, transparency may incur a cost on aposematism, with a trade-off between crypsis and aposematism. A possible explanation for the unexpected altitudinal distribution of transparency in Ithomiini species may be different predation pressures at different altitudes in tropical mountains (Roslin et al., 2017), which may favor crypsis over aposematism at higher altitudes, as observed in Andean arctiine moths or which conspicuousness decreases with height (Fiedler and Brehm, 2021). Therefore, the altitudinal distribution of color patterns in ithomiine may be primarily driven by predation rather than climatic conditions.

Conclusion

In Ithomiini, the altitudinal distribution of wing patterns is unlikely driven by thermal constraints, and goes against the predictions of the thermal melanism hypothesis. Transparent wings have a low absorption of wavelengths in the UV-Visible-NIR range and this comes at a cost regarding heating capacities. Conversely, transparency in these butterflies has probably evolved in response to selection incurred by predation. The results question the critical significance of wing color pattern for thermal adaptation in tropical ectotherms and lead the way to further investigations about metabolic and behavioral thermoregulation, and their relative contributions to climatic adaptation in these species.

4. Materials and Methods

Specimen sampling, climatic data and phylogeny

We studied wings of dried Ithomiini butterfly specimens, preserved in glassine envelopes, collected between 2005 and 2018 in south America. We focused on 42 species represented by two or more females to reduce the potential variance due to sexual differences (Table S1a, S1b). Measurements were taken on one hindwing and one forewing of each specimen. The selected species spanned a large range of wing coloration, from fully opaque to highly transparent, and are evenly distributed along the altitudinal gradient and across the phylogeny. GPS data on species distributions were obtained from a database that contains more than 3500 occurrences of specimens of the species studied here (Doré et al., 2022). We extracted the corresponding 19 climate variables (Table S8-9., Fig. S4) from WorldClim2 historical climatic data (Fick and Hijmans, 2017) with a resolution of 2.5 arc-minutes. Variables were averaged for each species. For the phylogenetic comparative analyses, we used the most comprehensive phylogeny of the Ithomiini to date (Chazot et al., 2019), which comprises 340 out of 393 extant taxa.

Spectrophotometry measurements

To quantify the extent to which wings transmit and absorb light, we performed spectrophotometry measurements on a spot of 5 mm of diameter on the hindwing and forewing of each specimen. For transparent species, the spot was in the proximal transparent patch. For opaque species, the spot was located on the center of the proximal zone of the wing. Since a wing can reflect and transmit a large proportion of the incident light, we measured both total (specular + diffuse) reflection and total (specular + diffuse) transmission spectra of each patch, with an Agilent CARY 5000 UV-Visible-NIR spectrophotometer, equipped with an integrating sphere, over the range 300-2500 nm, which includes ultraviolet (UV), Visible, and near-infrared (NIR) wavelengths. Wings were maintained in a black paper mask with an opening of 5 mm of diameter.

Spectra were measured relative to a dark (paper mask without the wings, light off) and to a white reference (Spectralon white diffuse reflectance standard), with the paper mask for reflection, nothing for transmission, light on. The resulting total absorption spectra were calculated for each wavelength λ as $\text{Absorption}(\lambda) = 1 - (\text{Transmission}(\lambda) + \text{Reflection}(\lambda))$ according to the law of conservation of energy (Fig. 4). The negative values due to the noise of the signal were fixed to zero with the function `fixneg` of the package `Pavo` in R (Maia et al., 2013), and each spectrum was smoothed with the `loess` function in R. We quantified mean absorption at different wavelength ranges, in the UV-Visible between 300-700 nm, in the NIR between 700-2500 nm, and the total 300-2500 nm. Mean transmission in the UV-Visible range 300-700 nm was measured as a proxy for animal-visible transparency. We divided the species

into two categories, transparent and opaque, based on a threshold of mean transmission at 35% in the UV-Visible, matching butterflies and predators' visual perception (Table S16).

Thermal measurements

To characterize and compare the wing thermal characteristics, we measured wing temperature kinetics following instant exposure to a flash light (Fig. 4B). We conducted stimulated heating measurements on one hindwing and one forewing for each specimen, with a Neewer professional photo system flash lamp N-300W set at maximum power, which emits over the UV-Visible-IR range. Wings were recorded by an infrared camera T650sc 2019 FLIR Systems equipped with a FLIR lens T197-915. Emissivity was set at 0.95. Wings were kept at a constant ambient temperature of 23°C prior to the experiment. We placed each single wing horizontally on a custom grid made of 3 human hair in a frame to minimize contact with any surface, 6 cm below the camera and the flash and 30 cm above a table covered with white paper, to avoid background interference with the measure. The flash lasted for 1/800 seconds. We started the recording a few seconds before activating the flash, and let the record run for 10 seconds. Each movie was recorded at 30 images.second⁻¹.

From the movies, we extracted the thermal kinetics - temperature as a function of time - of different regions of interest on the wings with FLIR ResearchIR Max software, and more specifically the excess temperature ΔT as the difference between the maximal temperature attained by the region of interest after the flash hits minus the temperature prior to the flash. We extracted mean ΔT of the overall wing surface, mean ΔT of the spot measured in spectrophotometry and finally ΔT of precise points of interest on each wings as follows: 6 points in the dark colored patches DP (black to brown colors), 6 points in light colored patches LP (yellow to orange colors and for opaque species only), 6 points in the transparent patches TP (for transparent species only), and 8 points on veins, assigned to a category depending on their color: dark veins DV (black to brown) and light veins LV (yellow to orange).

Image analysis

We used digital photography to quantify the proportion of dark pigmented surface on the full wings of all the specimens. Melanization in proximal zones of the wing can be of primarily importance in thermoregulation as the zone is closer to the thoracic muscles (Wasserthal, 1975). We also quantified the color proportion separately on the proximal and distal zone of each wing. We set the limit between the zones as the perpendicular line to the middle of the transect line from the wing insertion to its apex. We cropped the photos for each zone considered and the background was filled with green, a color ignored when calculating the color proportions. In R, we used the `getKMeanColors` function from `colordistance` package (Weller and Westneat, 2019) to sort the pixels in clusters according to their RGB values. We set the number of clusters to two, corresponding to mainly dark and brown, versus

transparent, or any other light colors in opaque wings. With ImageJ 1.52 (Schindelin et al., 2012) we computed the total surface of each wing in mm².

Statistical analyses

We explored various scenarios linking absorption, transmission, thermal capacities and climatic variables across the 42 species of the dataset (see supplementary materials for model details and results). Because specimens are not independent and the species are related through the phylogeny, we implemented for each test Bayesian mixed models with Markov Chain Monte Carlo, correcting for phylogenetic relatedness, using the R package MCMCglmm (Hadfield, 2010) and the phylogeny from (Chazot et al., 2019). We controlled for chain convergence visually and with the Heidelberg stationarity test diagnostic function of convergence heidel.diag from the R package coda (Plummer et al., 2006). To get accurate estimations of posterior distributions and avoid chain autocorrelation, we adjusted the iterations, burn-in and thinning for each model to ensure an effective sample size across all parameters of at least 1000. Models were run with a weakly informative prior for random effect and residual variances ($V = 1$, $\nu = 0.002$) not to constrain the exploration of parameter values. We selected the best model with a backward selection of fixed parameters based on Bayesian P-value of 0.05, within the 95% confidence interval excluding zero. In all the models, species identity was implemented as a random factor. We included all individuals in the models unless specified otherwise.

Acknowledgments

This work was funded by Clearwing ANR project (ANR-16-CE02-0012), HFSP project on transparency (RGP0014/2016). VO is recipient of a doctoral fellowship from the doctoral school FIRE, Learning Planet Institute. We are strongly grateful to authorities in Peru and Ecuador for providing collecting permits (Ecuador: 005-IC-FAU-DNBAPVS/MA, 019-IC-FAU-DNBAPVS/MA021; Peru: 236-2012-AG-DGFFS-DGEFFS, 201-2013-MINAGRI-DGFFS/DGEFFS, 002-2015-SERFOR-DGGSPFFS, 373-2017-SERFOR-DGGSPFFS; Gobierno Regional San Martín PEHCBM: 124-2016-GRSM/PEHCBM-DMA/EII-ANP/JARR). We warmly thank Raúl Aldaz, Paula Santacruz and Alexandre Toporov for providing help in the field, and Santiago Villamarín, the Museo de Historia Natural and Professor Gerardo Lamas for their support. We also thank Jean-Luc Bodnar and Kamel Mouhoubi from Reims University for technical advice with IR imaging, Pierre de Villemereuil for his help with Bayesian analysis, Maël Doré for bioclimatic and distribution data sharing, Willy Daney de Marcillac for his advice with optical measurements and Serge Berthier for inspiring discussion on clearwing butterflies.

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Chapter 1 - Appendix

Supplementary Figures and Tables

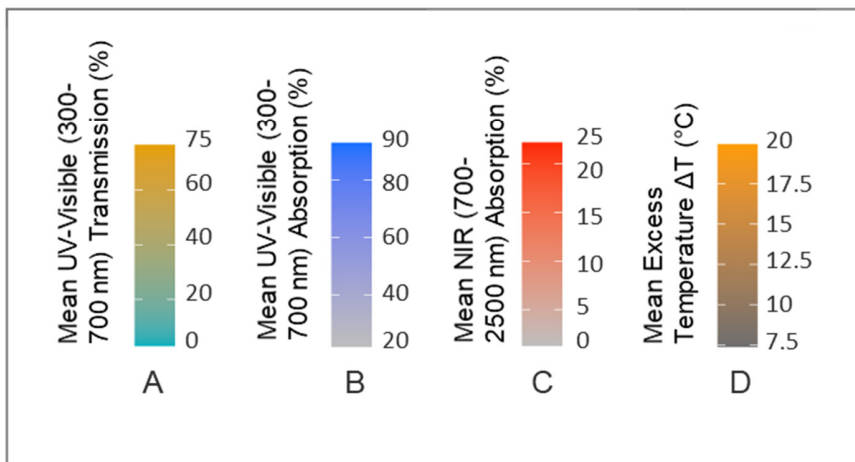
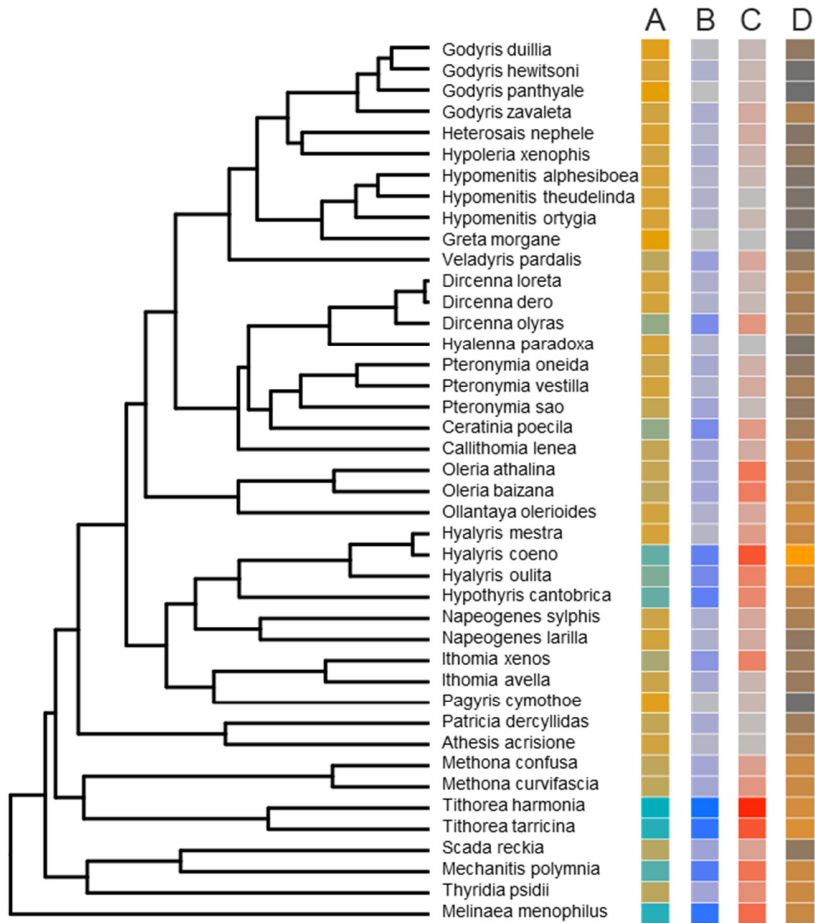


Figure S1. Legend next page.





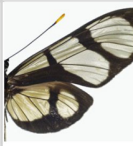
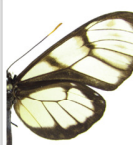


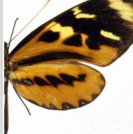
Figure S1. Distribution of optical and thermal traits along the phylogeny of the 42 ithomiine species studied, averaged for both wings and all the specimens of each species. A: Mean UV-visible (300 to 700nm) transmission for a spot of 5 mm of diameter on wing proximal zone. This value is used as a proxy for wing transparency. B: Mean UV-visible (300 to 700 nm) absorption for a spot of 5 mm of diameter on wing proximal zone. C: Mean NIR (700 to 2500 nm) absorption for a spot of 5 mm of diameter on wing proximal zone. D: Mean Excess Temperature ΔT ($T^{\circ}\text{final} - T^{\circ}\text{initial}$) in $^{\circ}\text{C}$ of the wings under a flash illumination.

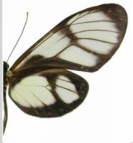

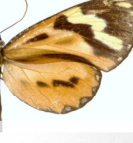


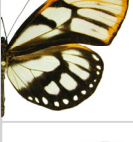





Table S1a. List of the species and the number of specimens per species studied. All specimens are females. The mean altitude of occurrence of each species is computed based on published data (1), and the mean transmission over the UV-visible range (300 to 700 nm) of the forewings and hindwings spots for each species are shown. The threshold for the division of the species in two groups - opaque or transparent- was set at 35% of transmission for both wings.



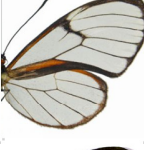

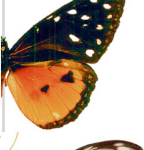
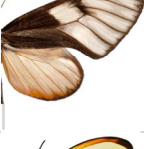

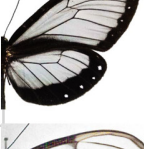



Genus, species	Number of specimens (females)	Mean altitude of occurrence in meters	Mean transmission % of the Forewing spot in the UV-Visible	Mean transmission % of the Hindwing spot in the UV-Visible	Category of the species
<i>Athesis acrisione</i>	2	752.00	63.14	57.70	Transparent
<i>Callithomia lenea</i>	4	601.37	55.34	51.31	Transparent
<i>Ceratinia poecila</i>	5	768.17	31.31	27.29	Opaque
<i>Dircenna dero</i>	4	515.31	67.46	58.86	Transparent
<i>Dircenna loreta</i>	3	780.39	66.04	59.21	Transparent
<i>Dircenna olyras</i>	2	894.08	32.27	27.27	Opaque
<i>Godyris duillia</i>	4	1404.10	73.00	71.68	Transparent
<i>Godyris hewitsoni</i>	4	2041.00	66.75	62.41	Transparent
<i>Godyris panthyale</i>	4	2023.83	80.80	71.00	Transparent
<i>Godyris zavaleta</i>	4	367.73	62.69	58.12	Transparent
<i>Greta morgane</i>	2	750.07	74.59	78.45	Transparent
<i>Heterosais nephele</i>	4	551.40	65.87	67.07	Transparent
<i>Hyalenna paradoxa</i>	2	2195.87	68.64	59.61	Transparent
<i>Hyaliris coeno</i>	3	831.05	26.57	1.96	Opaque
<i>Hyaliris mestra</i>	3	1447.95	61.78	66.41	Transparent
<i>Hyaliris oulita</i>	4	1208.42	29.53	13.61	Opaque
<i>Hypoleria xenophis</i>	4	710.00	60.13	61.87	Transparent
<i>Hypomenitis alphisiboea</i>	4	1545.38	60.24	70.71	Transparent
<i>Hypomenitis ortygia</i>	4	2030.49	71.43	59.42	Transparent
<i>Hypomenitis theudelinda</i>	4	2166.95	63.62	66.13	Transparent

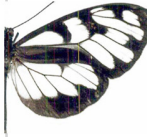






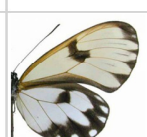


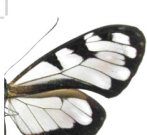
Genus, species	Number of specimens (females)	Mean altitude of occurrence in meters	Mean transmission % of the Forewing spot in the UV-Visible	Mean transmission % of the Hindwing spot in the UV-Visible	Category of the species
<i>Hypothyris cantabrica</i>	4	740.47	15.10	12.86	Opaque
<i>Ithomia avella</i>	4	2006.97	57.29	57.08	Transparent
<i>Ithomia xenos</i>	2	1319.83	36.96	41.94	Transparent
<i>Mechanitis polymnia</i>	5	596.00	13.35	7.99	Opaque
<i>Melinaea menophilus</i>	4	846.00	4.76	4.58	Opaque
<i>Methona confusa</i>	8	896.78	53.25	47.36	Transparent
<i>Methona curvifascia</i>	4	416.85	52.81	48.14	Transparent
<i>Napeogenes larilla</i>	4	2146.11	62.37	63.30	Transparent
<i>Napeogenes sylphis</i>	5	411.50	43.99	73.55	Transparent
<i>Oleria athalina</i>	4	2201.62	37.95	69.26	Transparent
<i>Oleria baizana</i>	4	2119.44	40.46	57.80	Transparent
<i>Ollantaya olerioides</i>	3	2029.27	57.74	65.48	Transparent
<i>Pagyris cymothoe</i>	4	1191.62	72.16	72.91	Transparent
<i>Patricia deryllidas</i>	2	2058.44	47.89	56.98	Transparent
<i>Pteronymia oneida</i>	4	2179.98	52.71	61.71	Transparent
<i>Pteronymia sao</i>	2	104.86	52.91	55.21	Transparent
<i>Pteronymia vestilla</i>	4	333.96	58.99	67.56	Transparent
<i>Scada reckia</i>	2	743.90	51.94	43.31	Transparent
<i>Thyridia psidii</i>	4	788.93	48.52	48.36	Transparent
<i>Tithorea harmonia</i>	7	543.08	3.78	2.16	Opaque
<i>Tithorea tarricina</i>	2	1285.44	2.09	6.92	Opaque
<i>Veladyris pardalis</i>	4	2036.77	59.91	38.54	Transparent

Table S1b. Overview of the species studied, ordered according to their mean altitude of occurrence.

Genus, species	Mean altitude of occurrence in meters	Category of the species	
<i>Pteronymia sao</i>	104.86	Transparent	
<i>Pteronymia vestilla</i>	333.96	Transparent	
<i>Godyris zavaleta</i>	367.73	Transparent	
<i>Napeogenes sylphis</i>	411.5	Transparent	
<i>Methona curvifascia</i>	416.85	Transparent	
<i>Dircenna dero</i>	515.31	Transparent	
<i>Tithorea harmonia</i>	543.08	Opaque	
<i>Heterosais nephele</i>	551.4	Transparent	
<i>Mechanitis polymnia</i>	596	Opaque	

<i>Callithomia lenea</i>	601.37	Transparent	
<i>Hypoleria xenophis</i>	710	Transparent	
<i>Hypothesis cantobrica</i>	740.47	Opaque	
<i>Scada reckia</i>	743.9	Transparent	
<i>Greta morgane</i>	750.07	Transparent	
<i>Athesis acrisione</i>	752	Transparent	
<i>Ceratinia poecila</i>	768.17	Opaque	
<i>Dircenna loreta</i>	780.39	Transparent	
<i>Thyridia psidii</i>	788.93	Transparent	
<i>Hyalyris coeno</i>	831.05	Opaque	
<i>Melinaea menophilus</i>	846	Opaque	

<i>Dircenna olyras</i>	894.08	Opaque	
<i>Methona confusa</i>	896.78	Transparent	
<i>Pagyris cymothoe</i>	1191.62	Transparent	
<i>Hyalyris oulita</i>	1208.42	Opaque	
<i>Tithorea tarricina</i>	1285.44	Opaque	
<i>Ithomia xenos</i>	1319.83	Transparent	
<i>Godyris duillia</i>	1404.1	Transparent	
<i>Hyalyris mestra</i>	1447.95	Transparent	
<i>Hypomenitis alphasiboea</i>	1545.38	Transparent	
<i>Ithomia avella</i>	2006.97	Transparent	
<i>Godyris panthyle</i>	2023.83	Transparent	

<i>Ollantaya olerioides</i>	2029.27	Transparent	
<i>Hypomenitis ortygia</i>	2030.49	Transparent	
<i>Veladyris pardalis</i>	2036.77	Transparent	
<i>Godyrus hewitsoni</i>	2041	Transparent	
<i>Patricia deryllidas</i>	2058.44	Transparent	
<i>Oleria baizana</i>	2119.44	Transparent	
<i>Napeogenes larilla</i>	2146.11	Transparent	
<i>Hypomenitis theudelinda</i>	2166.95	Transparent	
<i>Pteronymia oneida</i>	2179.98	Transparent	
<i>Hyalenna paradoxa</i>	2195.87	Transparent	
<i>Oleria athalina</i>	2201.62	Transparent	

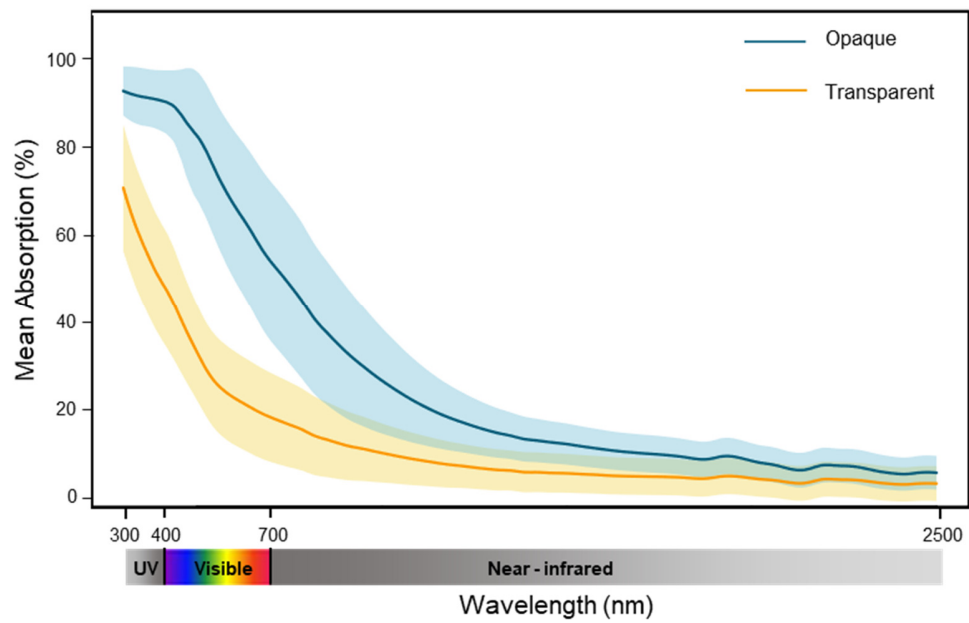


Figure S2. Mean absorption spectra and 95% standard deviation of opaque and transparent wing spots from 300 to 2500 nm. Absorption over the whole spectrum is significantly correlated with transmission in the UV-Visible range, as tested by the Bayesian phylogenetic mixed model (see Table S2) (N opaque species = 9, N transparent species = 33).

Table S2. Retained Bayesian mixed models correcting for phylogenetic relatedness of the optical relationship between absorption and transmission spectra of the wing spots at different ranges, after backward selection of fixed parameters based on Bayesian P-value criteria. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Mean absorption of the wing spot over 300 to 2500 nm	(Intercept)	13.992	11.873	16.081	156309.039	< 0.001 ***
	Mean transmission over 300 to 700 nm	-8.450	-9.124	-7.790	102746.658	< 0.001 ***
	phylogenetic variance	5.953	0.000	10.327	5927.130	
	Species identity	0.385	0.000	2.097	1548.627	
	residual variance	7.621	6.380	8.975	183185.405	
Mean absorption of the wing spot over 700 to 2500 nm	(Intercept)	6.964	4.433	9.423	361960.677	< 0.001 ***
	Mean transmission over 300 to 700 nm	-5.326	-6.091	-4.540	108436.277	< 0.001***
	phylogenetic variance	8.348	0.000	14.321	7731.567	
	Species identity	0.490	0.000	2.689	1436.113	
	residual variance	10.727	8.941	12.581	353377.654	
Mean absorption of the wing spot over 700 to 2500 nm	(Intercept)	6.038	2.134	10.106	32631.740	< 0.01**
	Mean absorption over 300 to 700 nm	5.146	3.146	7.061	31417.877	< 0.001***
	phylogenetic variance	8.328	3.367	14.214	4269.801	
	Species identity	0.239	0.000	1.188	3244.589	
	residual variance	10.096	8.402	11.851	32559.210	
Other fixed parameters not retained : Type of wing (opaque > transparent); Mean absorption over 300 to 700 nm * Type of wing						

Table S3. Bayesian mixed models correcting for phylogenetic relatedness of the relationship between the thermal properties of the wing spots and their optical properties at different ranges. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Excess temperature ΔT of the wing spot	(Intercept)	11.932	10.033	13.761	19500.000	< 0.001 ***
	Mean transmission over 300 to 700 nm	-1.546	-1.919	-1.167	13067.170	< 0.001 ***
	phylogenetic variance	5.232	2.449	8.410	3153.368	
	Species identity	0.128	0.000	0.597	1979.854	
	residual variance	1.586	1.331	1.877	18984.158	
	(Intercept)	12.364	10.100	14.534	18828.512	< 0.001 ***
	Mean absorption over 300 to 2500 nm	0.983	0.671	1.280	14300.000	< 0.001 ***
	phylogenetic variance	7.256	3.170	11.700	2900.000	
	Species identity	0.267	0.000	1.190	1860.000	
	residual variance	1.612	1.350	1.901	19060.100	
	(Intercept)	12.548	10.000	14.954	19500.000	< 0.001 ***
	Mean absorption over 700 to 2500 nm	0.596	0.351	0.864	14643.765	< 0.001 ***
	phylogenetic variance	8.961	3.520	14.847	1935.934	
	Species identity	0.537	0.000	1.996	1608.264	
	residual variance	1.658	1.390	1.958	17949.578	

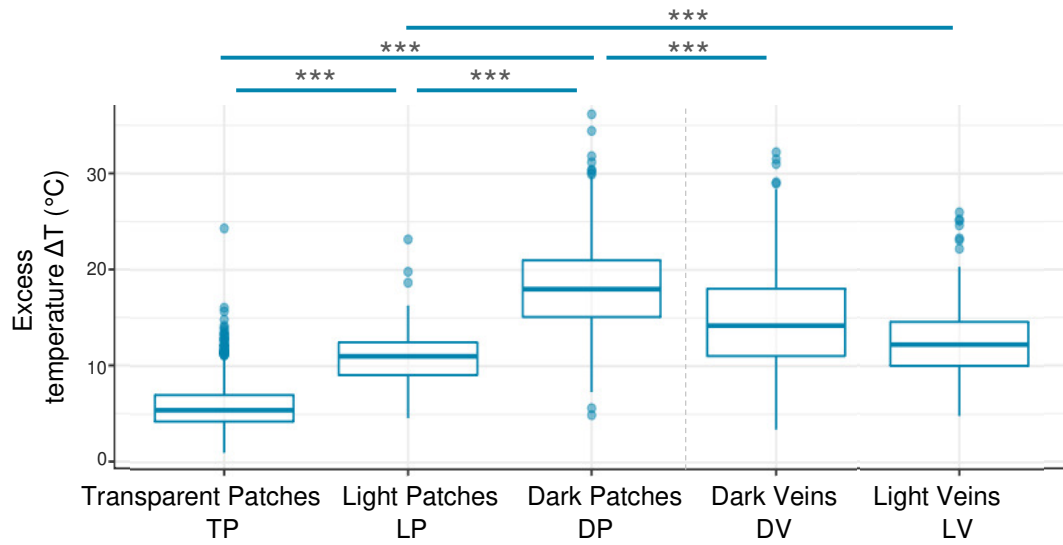


Figure S3. Excess Temperature ΔT ($T^{\circ}\text{final} - T^{\circ}\text{initial}$) of different color patches and veins on the wings, under a flash illumination, extracted from the thermal imaging. The tested pairs shown here correspond to the Bayesian phylogenetic mixed model in the tables S4 to S7. The significance of the effects corresponds to an estimated Bayesian P-value (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) (N TP= 1407, N LP= 408, N DP=1829, N DV=2117, N LV= 325) associated to a 95% credibility interval excluding zero.

Table S4. Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the mean excess temperature ΔT of the Dark patches DP of butterfly and the Lighter LP and Transparent patches TP, and the zone of the wing, proximal P or distal D. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Excess temperature ΔT	(Intercept)	20.212	18.400	22.076	591933.290	< 0.001 ***
	Patch color DP>(LP, TP)	11.775	12.000	11.507	592594.820	< 0.001 ***
	Zone (P>D)	-1.253	-1.520	-0.990	596000.000	< 0.001 ***
	Patch color DP>(LP, TP) x Zone (P>D)	-1.290	-0.915	-1.667	592931.730	< 0.001 ***
	phylogenetic variance	5.170	2.750	7.975	62364.570	
	Species identity	0.109	0.000	0.485	35697.100	
	residual variance	8.381	8.000	8.769	593640.220	

Table S5. Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the mean excess temperature ΔT of patches and patch color (Light patches LP and Transparent patches TP), and wing zone (proximal P or distal D). Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Excess temperature ΔT	(Intercept)	10.067	8.630	11.477	45300.000	< 0.001 ***
	Patch color (LP>TP)	2.813	1.609	4.080	9450.000	< 0.001 ***
	Zone (P>D)	1.683	2.020	1.340	49500.000	< 0.001 ***
	Patch color (LP>TP) x Zone (P>D)	2.055	2.433	1.670	49500.000	< 0.001 ***
	phylogenetic variance	1.979	0.368	4.029	2602.930	
	Species identity	0.817	0.000	1.624	4488.541	
	residual variance	2.925	2.733	3.118	49500.000	

Table S6. Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the mean temperature excess ΔT and Dark patches DP vs Dark Veins DV, and wing zone (proximal P or distal D). Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Excess temperature ΔT	(Intercept)	19.570	17.441	21.620	48100.00	< 0.001 ***
	Patch color (DP>DV)	5.517	5.870	5.163	49500.00	< 0.001 ***
	Zone (P>D)	-1.255	-1.610	-0.918	49500.00	< 0.001 ***
	Patch color (DV>DP) x Zone (P>D)	-3.491	-3.010	-3.958	49500.00	< 0.001 ***
	phylogenetic variance	5.896	1.310	11.043	3380.00	
	Species identity	1.567	0.000	3.357	5550.00	
	residual variance	14.068	13.500	14.706	49500.00	

Table S7. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the mean temperature excess ΔT and light patches LP vs the Light Veins LV, and wing zone (proximal P or distal D), after backward selection of fixed parameters based on Bayesian P-value criteria. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Excess temperature ΔT	(Intercept)	8.514984	6.030	11.142413	91944.511	< 0.001 ***
	Patch color (LV> LP)	3.513077	3.050	3.980312	464070.148	< 0.001 ***
	Zone (P>D)	1.33338	0.947	1.710718	596000	< 0.001 ***
	phylogenetic variance	7.346833	0.000	15.386159	4531.189	
	Species identity	1.560804	0.000	7.593318	1492.881	
	residual variance	5.784633	5.190	6.395479	596000	
	Other fixed parameters not retained in the best model : Patch color (LV> LP) x Zone (P>D)					

Table S8. Coordinates of WorldClim variables on the climatic principal component PC1.

WorldClim Variables	Coordinates on PC1
Min Temperature of Coldest Month	0.937
Mean Temperature of Driest Quarter	0.926
Mean Temperature of Warmest Quarter	0.915
Annual Mean Temperature	0.908
Mean Temperature of Wettest Quarter	0.901
Mean Temperature of Coldest Quarter	0.895
Max Temperature of Warmest Month	0.885
Precipitation of Warmest Quarter	0.858
Precipitation of Wettest Quarter	0.776
Annual Precipitation	0.757
Precipitation of Wettest Month	0.759
Isothermality (Mean Diurnal Range/Temperature Annual Range) (×100)	-0.549
Precipitation of Driest Quarter	0.457
Temperature Seasonality (standard deviation ×100)	0.432
Precipitation of Driest Month	0.443
Mean Diurnal Range (Mean of monthly (max temp - min temp))	-0.417
Precipitation of Coldest Quarter	0.337
Precipitation Seasonality (Coefficient of Variation)	0.107
Temperature Annual Range (Max Temperature of Warmest Month - Min Temperature of Coldest Month)	0.011

Table S9. Coordinates of WorldClim variables on the climatic principal component PC2.

WorldClim Variables	Coordinates on PC2
Precipitation of Coldest Quarter	0.907
Temperature Annual Range (Max Temperature of Warmest Month - Min Temperature of Coldest Month)	-0.833
Precipitation of Driest Quarter	0.812
Precipitation of Driest Month	0.802
Annual Precipitation	0.608
Precipitation Seasonality (Coefficient of Variation)	-0.607
Mean Diurnal Range (Mean of monthly (max temp - min temp))	-0.537
Isothermality (Mean Diurnal Range/Temperature Annual Range) (×100)	0.507
Temperature Seasonality (standard deviation ×100)	-0.452
Precipitation of Wettest Quarter	0.373
Max Temperature of Warmest Month	-0.398
Mean Temperature of Wettest Quarter	-0.367
Precipitation of Wettest Month	0.322
Mean Temperature of Warmest Quarter	-0.344
Annual Mean Temperature	-0.322
Mean Temperature of Coldest Quarter	-0.291
Precipitation of Warmest Quarter	0.259
Mean Temperature of Driest Quarter	-0.237
Min Temperature of Coldest Month	-0.165

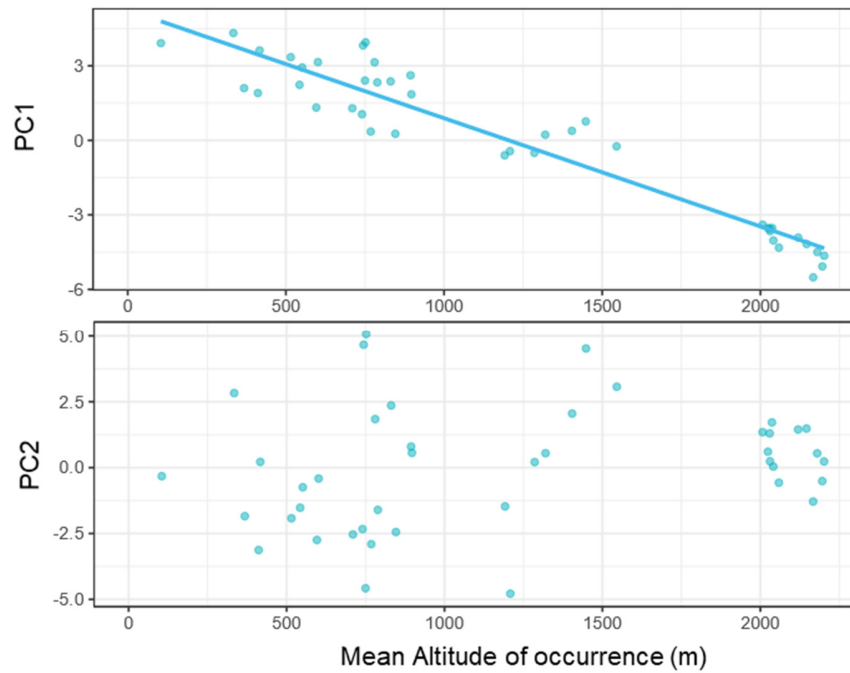


Figure S4. Predicted coordinates of the studied species on the climatic principal components as a function of their mean altitude of occurrence. The relationship is significant for PC1 (Pearson's product-moment correlation test: $R = -0.95$, $n = 42$, $p < .0001$) but not for PC2 (Pearson's product-moment correlation test: $R = 0.24$, $n = 42$, $p = 0.12$) ($N = 42$).

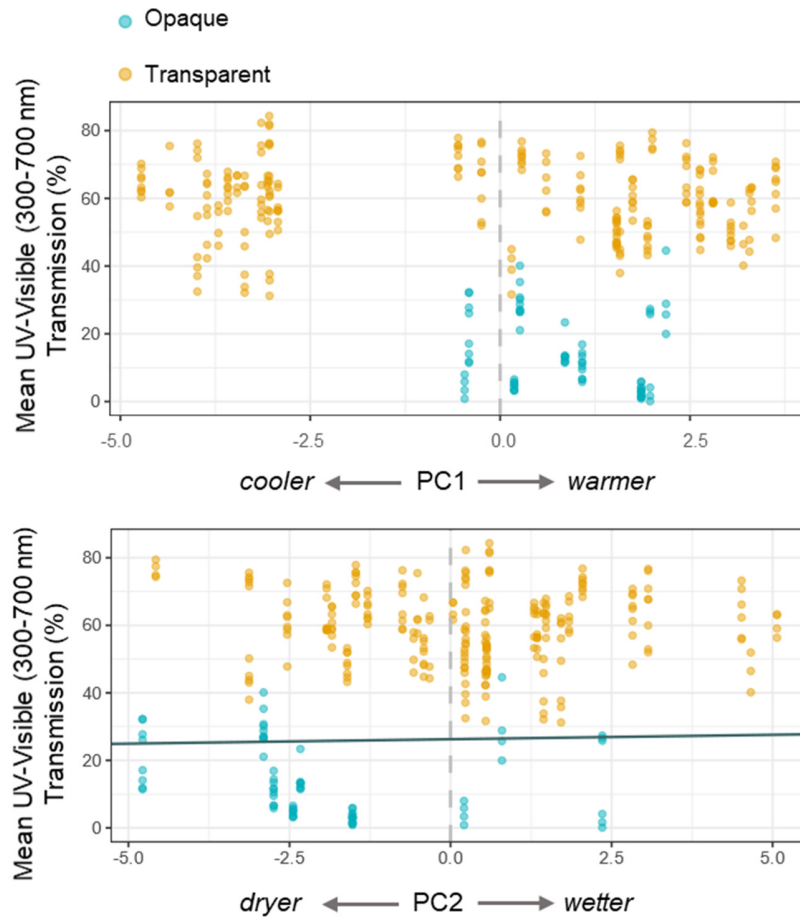


Figure S5. Wing mean transmission (%) in the UV-Visible range (300-700 nm) as a function of climatic principal components PC1 and PC2. The relationship is not significant for PC1, and is significant for PC2 (see Table S10), and the solid line is drawn from the prediction of the Bayesian phylogenetic mixed model (N opaque = 72, N transparent = 235).

Table S10. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the mean transmission of the wing spots over the range 300 to 700 nm and climatic variables, after backward selection of fixed parameters based on Bayesian P-value criteria. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Mean transmission of the wing spot over 300 to 700 nm	(Intercept)	38.144	22.690	53.214	9323.783	< 0.001***
	PC2	4.698	0.202	9.504	19500.000	0.048 *
	phylogenetic variance	291.857	63.653	570.576	1433.784	
	Species identity	144.982	35.479	275.447	1282.366	
	residual variance	60.095	49.943	70.440	19500.000	
Other fixed parameters not retained: PC1; PC1 x PC2						

Table S11. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the mean absorption of the wing spots over the range 300 to 2500 m and the climatic variables, after backward selection of fixed parameters based on Bayesian P-value criteria. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Mean absorption of the wing spot over 300 to 2500 nm	(Intercept)	18.187	12.226	24.068	14519.316	< 0.001***
	phylogenetic variance	45.574	12.876	87.789	1597.004	
	Species identity	14.485	0.001	30.014	2264.057	
	residual variance	18.862	15.813	22.190	19500.000	
Fixed parameters not retained: PC1; PC2; PC1 x PC2						

Table S12. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the mean absorption of the wing spots over the range 700 to 2500 m and the climatic variables, after backward selection of fixed parameters based on Bayesian P-value criteria. Fixed effect estimates with 95% credibility excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Mean absorption of the wing spot over 700 to 2500 nm	(Intercept)	9.835	5.645	14.080	18746.162	< 0.001***
	phylogenetic variance	25.140	10.090	43.846	1973.388	
	Species identity	2.197	0.000	7.761	1023.411	
	residual variance	16.082	13.416	18.877	15939.104	
	Fixed parameters not retained: PC1; PC2; PC1 x PC2					

Table S13. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the mean temperature excess ΔT of the full wing and the climatic variables, after backward selection of fixed parameters based on Bayesian P-value criteria. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Mean excess temperature ΔT of the full wing	(Intercept)	13.317	11.100	15.647	18800.000	< 0.001***
	PC1	0.810	0.198	1.421	17912.730	< 0.05*
	Phylogenetic variance	7.464	2.770	12.917	1750.074	
	Species identity	0.828	0.000	2.324	1816.949	
	Residual variance	2.256	1.890	2.658	18079.378	
Other fixed parameters not retained: PC2; PC1 x PC2; category of the species (transparent or opaque); area of the wing; identity of the wing (forewing or hindwing)						

Table S14. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the proportion of dark patches on the surface of each wing and climatic variables, after backward selection of fixed parameters based on Bayesian P-value criteria. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold,

associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Proportion of dark patches on the wings	(Intercept)	50.492	40.770	59.900	48300	< 0.001 ***
	PC1	4.558	1.599	7.530	11300	< 0.01 **
	Wing identity Forewing>Hindwing	-3.886	-6.079	-1.731	49900	< 0.001 ***
	phylogenetic variance	123.971	27.181	242.779	2070	
	Species identity	22.540	0.000	68.837	1270	
	Residual variance	92.840	77.400	109.543	29163.515	
Other fixed parameters not retained: PC2; PC1 x PC2; category of the species (transparent or opaque); area of the wing						

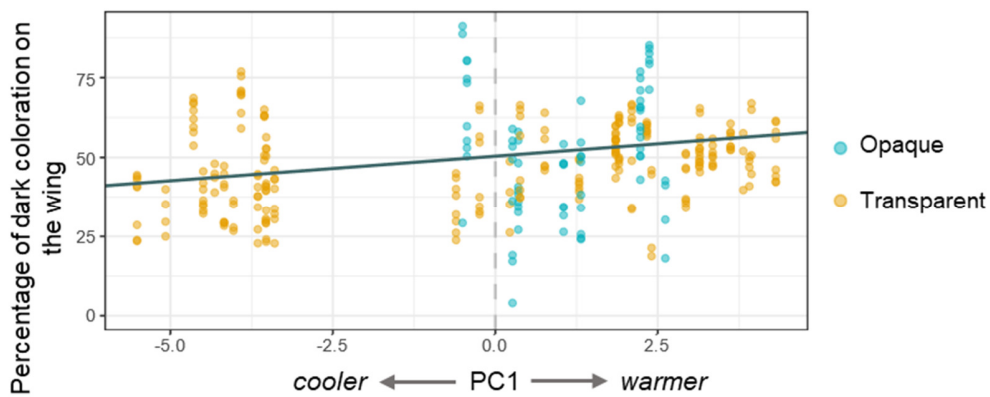


Figure S6. Proportion of dark coloration on the surface of butterfly wings as a function of climatic principal component PC1. The relationship is significant and the solid line is drawn from the prediction of the Bayesian phylogenetic mixed model (see Table S14) (N opaque = 72, N transparent = 235).

Table S15. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the proportion of dark patches on the surface of each wing among transparent species as a function of climatic variables, after backward selection of fixed parameters based on Bayesian P-value criteria. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Proportion of dark patches on transparent wings	(Intercept)	49.073	39.100	58.651	49000.000	< 0.001 ***
	PC1	5.219	2.780	7.659	38812.461	< 0.001 ***
	Phylogenetic variance	104.112	44.500	180.245	5872.326	
	Species identity	2.086	0.000	11.100	3568.089	
	Residual variance	65.149	53.200	78.500	45847.698	
Other fixed parameters not retained: PC2; PC1 x PC2; area of the wing; identity of the wing (forewing or hindwing)						

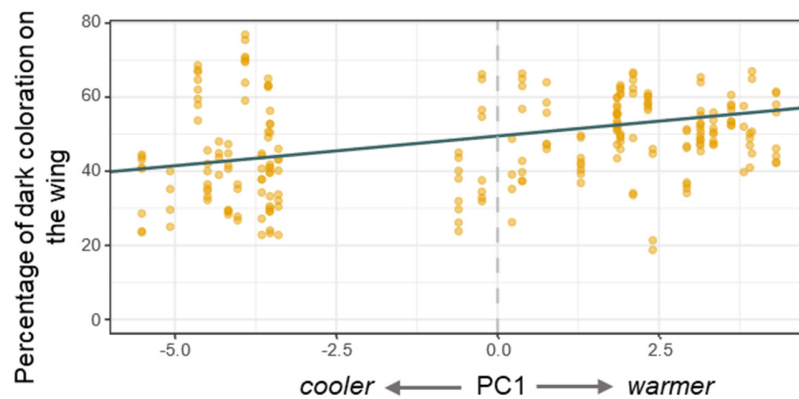


Figure S7. Proportion of dark coloration on the surface of butterfly wings among transparent species only as a function of climatic principal component PC1. The relationship is significant and the solid line is drawn from the prediction of the Bayesian phylogenetic mixed model (see Table S15) (N = 235).

Table S16. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the proportion of colored patches in the proximal zone of each wing among transparent species as a function of climatic variables, after backward selection of fixed parameters based on Bayesian P-value criteria. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factor for which the parameters were not significant is listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Proportion of dark patches in the proximal zone of transparent wings	(Intercept)	53.271	42.800	63.465	1280000	< 0.001 ***
	PC1	4.676	1.490	7.851	30600	< 0.001 ***
	PC2	3.292	0.641	5.967	984000	< 0.05*
	Wing identity forewing > hindwing	-16.474	-18.900	-14.054	1380000	< 0.001 ***
	Phylogenetic variance	104.876	0.000	206.986	10000	
	Species identity	17.428	0.000	81.513	3413.408	
	Residual variance	89.498	72.400	107.592	1092255.180	
	Other fixed parameters not retained: PC1 x PC2					

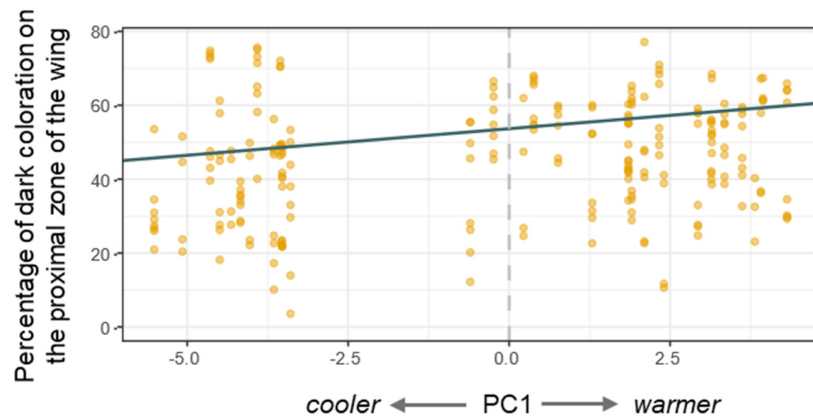


Figure S8. Proportion of dark coloration on the surface of proximal zones of butterfly wings among transparent species as a function of climatic principal component PC1. The relationship is significant. The solid line is drawn from the prediction of the Bayesian phylogenetic mixed model (see Table S16) (N = 235).

Table S17. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between the total area of the hindwing plus forewing of each butterfly and the climatic variables. Retained Bayesian mixed model corrected for phylogenetic relatedness, after backward selection of fixed parameters based on Bayesian P-value criteria. Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian P-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factor for which the parameters were not significant is listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian P-value
Total surface of the hindwing plus forewing of each butterfly	(Intercept)	759.086	519.548	997.923	59383.942	< 0.001***
	phylogenetic variance	86397.084	44978.920	135603.575	4361.406	
	Species identity	1206.417	0.000	7524.155	1223.185	
	residual variance	5080.964	3795.435	6458.049	59000.000	
	Fixed parameters not retained: PC1; PC2; PC1 x PC2					

Chapter 2. Thermal response in Andean butterfly communities: buffering and thermal tolerance vary with elevation

Authors

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Abstract

The Ecuadorian Andes is one of the richest terrestrial biodiversity hotspots, with a strong altitudinal variation in abiotic factors, and a parallel strong altitudinal species turnover. Ectotherm activity is constrained by environmental conditions, notably by ambient temperature. Their body temperature is the result of morphological, physiological and behavioral mechanisms. Their ability to buffer ambient temperature and to tolerate thermal stress situations is directly related to their distribution. Here, we compared the capacity for thermal buffering of low and high-altitude butterflies species of the tribe Ithomiini (Nymphalidae), and explored their thermal tolerances under cold and heat stress conditions. By taking phylogenetic relatedness into account in the analysis, we showed that the variations observed are due to environmental adaptation and not only shared ancestry. The two altitudinal communities showed divergent patterns of buffering strategy. The lowland species were generally thermoconforming. On the other hand, highland species, despite not showing a clear buffering pattern, did not have a strong thermoregulatory ability. We found that highland species resisted longer to cold stress, but were less resilient to heat stress than species of the lowland community. Our results highlight thermal sensitivity of highland populations, and raises concerns about the impacts of climate change on Neotropical mountains, which are among the most rapidly warming regions in the world.

1. Introduction

Tropical regions have a narrow annual temperature range, and temperature segregation with altitude is more marked in the tropics than in temperate regions (Janzen, 1967). Tropical species are then expected to be more thermal and elevational specialists as they experience low temperature variations, with narrow thermal niches, and a weak dispersal potential along elevational gradient (Perez et al., 2016; Polato et al., 2018). It ultimately leads to high speciation rate but also high vulnerability to temperature changes (Deutsch et al., 2008; Ghalambor, 2006), making tropical systems among the most vulnerable to climate change (Williams et al., 2007). In the tropics, mountains offer the greatest variation in abiotic factors, and air temperature changes at a rate of about 0.5 to 0.6°C per 100 m of altitude (Osborne, 2012). This makes mountain communities a key study system to investigate adaptations to climate, especially in ectotherms, which are highly dependent on the environmental temperature to be active.

Ectotherms need to maintain their body temperature within a certain range to achieve activity. Their tolerances to cold or heat stress exposure have limits beyond which conditions are unfavorable (Huey and Stevenson, 1979; Angilletta, 2009). These tolerances are mostly linked to climatic conditions, supporting the assumption that they are ecologically relevant to thermal adaptation (Hoffmann et al., 2002; Khazan et al., 2022). Their ability to adjust their body temperature in response to varying surrounding ambient conditions can be achieved through strategies involving behavioural, physiological and morphological traits (Angilletta, 2009). Animals that are able to buffer and compensate for variations in ambient temperature are called thermoregulators, while animals of which body temperature mostly follows that of the environment are called thermoconformers (Bladon et al., 2020; Huey and Slatkin, 1976).

Studies linking tropical ectotherm buffering and tolerance responses to climatic conditions is scarce in the literature. Tropical species have a narrower range of thermal tolerance, and generally lower maximum and higher minimum thermal limits, compared to what is described for high latitude species (Addo-Bediako et al., 2000; Clusella-Trullas et al., 2011; Polato et al., 2018; Sunday et al., 2011). Similarly, thermal tolerance follows an altitudinal gradient, mostly driven by an increased the cold tolerance in altitude, with little variation of heat tolerance along the gradient (Sunday et al., 2019). Some studies on tropical species, however, found that population tolerance to high temperatures decreases with elevation, as in rolled-leaf beetles (García-Robledo et al., 2016) and *Heliconius* butterflies (Montejo-Kovacevich et al., 2020).

In a multi-taxon study of butterflies in Panama, a higher buffering ability has been found in darker and larger species, but small species are more able to tolerate heat stress (Ashe-Jepson et al., 2023a), yet, their average buffering ability is higher than that of European communities (Laird-Hopkins et al., 2023). However, these last two studies have not controlled for phylogenetic relationships between species, which limits the opportunity to interpret the

results in terms of adaptation. Hence, there is a need for an interspecific study investigating the questions of buffering ability and thermal tolerance in tropical communities along an elevational gradient, with a proper phylogenetic control.

Here we explore this question in Ithomiini, a clade of butterflies of about 400 species widespread in the Neotropics, and most specifically in the Andes, where their diversity peaks (Doré et al., 2022). Ithomiine butterflies can be found from sea level up to 3000 meters (Chazot et al., 2019; Doré et al., 2022), and species, which are mostly forest dwellers (Willmott et al., 2017), are highly segregated along altitudinal gradients (Chazot et al., 2014). Surprisingly, high-altitude ithomiine species are much less melanic than low altitude species (Ossola et al., 2023), at odds with the thermal melanism hypothesis which posits that ectotherms inhabiting cold environments should be darker than those living in warmer climates (Clusella Trullas et al., 2007). Moreover, many species have partially transparent wings, which is a thermal disadvantage in terms of light and heat absorption (Ossola et al., 2023), and the proportion of clearwing species increases with altitude. Adaptation to altitude in Ithomiini may therefore lie in mechanisms other than dark colorations, such as physiological and behavioural adaptations, making it a particularly relevant group to study buffering ability and thermal tolerance. Here we study two communities of ithomiine butterflies at high and low altitudes in the Ecuadorian Andes, and determine whether there are physiological differences in response to temperature. We compare, on an interspecific scale and on a community scale, whether ithomiine species differ in (i) their buffering ability by measuring their thoracic temperature in their natural environment (Kleckova et al., 2014), and (ii) in their resistance to controlled cold and heat stress conditions (Khazan et al., 2022; Montejo-Kovacevich et al., 2020). Within a phylogenetic framework, we aim to test two predictions:

1) High elevation species should, on average, have a better buffering ability than low elevation species. Indeed, to compensate for low temperatures, species living in colder climates, in the highlands, should be able to elevate their body temperature relative to ambient temperature more than those at lower altitudes, which should conform more to ambient conditions.

2) Species sharing the same altitudinal niche should have similar thermal tolerance. High-altitude species should, on average, be more resistant to cold and more sensitive to heat than low-altitude species.

To test these predictions, we first performed linear regressions or mixed models, without phylogenetic correction, which enabled us to detect significant associations between buffering abilities, thermal tolerances and altitude, regardless of the cause of such associations. We then performed phylogeny-controlled analyses, which enabled us to assess whether the associations detected in the previous step entirely stemmed from phylogenetic relatedness or not. In the first case (phylogeny explains everything), associations of buffering abilities or thermal tolerances with altitude could be the result of random evolution along the phylogeny (i.e. only lineages with a propensity for buffering or tolerate extreme temperatures could colonize a

given altitude). In the second case (phylogeny does not explain everything), those associations can be interpreted as the result of genetic or plastic adaptation.

2. Material and Methods

Study sites and butterflies

We studied 2 natural communities of ithomiine butterflies, at high and low altitudes, in the Napo province of Ecuador. Sampling was carried out in December 2021 and December 2022. We measured 434 individuals from 20 species in the highland community and 458 Individuals from 45 species in the lowland communities, amounting to a total of 65 species and 892 individuals (Table S1). The lowland sites, in Archidona, Rio Pusuno and Tena, were on average at 530 meters of altitude, and the highland sites at 2120 meters of altitude, around the Yanayacu station, Cosanga. To describe the thermal characteristics of the collection sites, we extracted bioclimatic data for all capture sites from the published WorldClim2 dataset (Fick and Hijmans, 2017), with a resolution of 2.5 arc-minutes based on the sites coordinates (Table 1).

Table 1. Thermal characteristics of the collection sites of the populations from which individuals were collected. The data were extracted from the published WorldClim2 dataset (Fick and Hijmans, 2017), with a resolution of 2.5 arc-minutes based on the sites coordinates.

Collection site	Archidona	Tena	Rio Pusuno	Yanayacu
longitude	-77.800748	-77.8624475	-77.598851	-77.891418
latitude	-0.891977	-0.95212979	-1.020119	-0.601062
Altitude (m)	609	593	397	2120
Annual Mean Temperature (°C)	22.8	22.5	23.8	16
Mean Diurnal Range (Mean of monthly (max temperature - min temperature)) (°C)	9.9	9.9	9.9	12.3
Max Temperature of Warmest Month (°C)	28.6	28.2	29.5	23
Min Temperature of Coldest Month (°C)	17.4	17	18.3	9.2
Temperature Annual Range (Max Temperature of Warmest Month-Min Temperature of Coldest Month) (°C)	11.2	11.2	11.2	13.8

Buffering ability measurements

We sampled ithomiine butterflies in their natural habitat with insect nets. At the moment of capture, we immediately applied a thermocouple type K probe against the thorax of the butterflies through the mesh without handling or touching it, and registered its external thoracic temperature (Kleckova et al., 2014). External thoracic temperature is a good proxy of internal body temperature in butterflies as both do not differ a lot and the variance in this difference is very low (Nève and Hall, 2016). We recorded the ambient temperature at the instant and exact place of capture, noted the activity of the butterfly (with the following categories: courtshiping, feeding, flying, resting, basking). Individuals collected were then kept alive in glassine envelopes and brought to the lab for the experiments on the same or the very next day following the capture. They were fed *ad libitum* with sugary water (10%).

Thermal tolerance: cold and heat shock tolerance experiments

Back to the lab, a given individual performed first a cold and then a heat static stress experiment. In these static assays, the butterflies are exposed to a given temperature. The rate of injury accumulation is constant over time, and the time at which individuals reach a state of knock-out is recorded (Jørgensen et al., 2019). Individuals were fed *ad libitum* with sugary water (10%) before and in between each test to avoid dehydration effects on behavioural response. Cold and heat shock tolerance were measured with knock out tests, which enable to assess the tolerance to extreme temperature shocks without acclimation (Ashoff and Schmitt, 2014; Khazan et al., 2022; Montejo-Kovacevich et al., 2020).

First, we performed a cold shock experiment, during which the butterfly was placed in a glass jar (320 cm³) with paper at the bottom for it to stand on, along with a thermocouple type K probe to monitor the temperature experienced by the butterfly. The temperature inside the jar was maintained at 5°C throughout the whole test, and was manually adjusted as the jar was placed in or out a container with ice and water as needed. The butterfly was left in the jar for 5 minutes and we monitored the time until we noticed knock out, i.e when the butterfly was unable to move or stand on its legs. After 5 minutes, we took it out of the jar (i. e., the butterfly was placed at room temperature) and recorded the time until which the butterfly retrieves the capacity to stand on its legs, noted as the recovery time.

The heat shock trial was performed on individuals that went through a full recovery to cold shock, on the same day or the very next day. Each butterfly was placed in a similar glass jar, introduced in hot water bath ClearLine 062826CL to aim an inside temperature of 39°C throughout the whole trial. As in the cold test, temperature within the jar was constantly monitored and adjusted by temporarily removing the jar from the water bath if needed. We added wet cotton at the bottom to avoid dehydration. The time until knock out was monitored, and the butterfly was immediately removed from the jar. Butterflies were then frozen and dried to be weighted at the precision of 0.001g.

Statistical analyses

Differences in butterfly activity at capture between the two communities was tested with a Pearson's chi square test, and their flight temperature condition with a Welsh t-test.

To explore butterfly buffering ability and compare them between the two communities, we fitted simple linear regression models of T_{body} against T_{air} of the 17 species for which we had temperature measures for 10 or more individuals. We extracted the slope of the relationship, its intercept and confidence interval, set at 0.95. The closest the slope to 1, the more thermoconformer is the species or group tested, while a slope close to 0 would indicate a thermoregulatory ability (see : Ashe-Jepson et al., 2023a; Bladon et al., 2020; Huey and Slatkin, 1976).

We independently explored the effect of the altitudinal community on the air temperature at which the butterflies were collected in flight, the buffering ability, and the thermal tolerance to cold and heat stress.

To test whether the observed variations are due to the altitudinal community, we fitted linear mixed models with the package nlme (Pinheiro et al., 2023), with backward variable selection based on a p-value fixed at 0.05. Weight and sex were added as a variable in the models, where applicable (see result tables).

In the cold and heat shock tolerance tests, the mean temperature and the standard deviation of the temperature in the jar during the trials were added as fixed factors in the statistical analysis. In the tests for the cold stress recovery, we also added as a fixed factor the temperature difference between the ambient temperature experienced by the butterfly during recovery, with the average temperature experienced by other butterflies during recovery of the same altitudinal community.

To assess whether the effects detected in the first series of tests were entirely explained by phylogenetic relatedness or not, we performed the same analyses, but controlling for the phylogeny. We used the most comprehensive phylogeny to date (Chazot et al., 2019), which comprises 339 out of 396 extant ithomiine taxa, including all the species of this study. When the data concerned individual measurements, we implemented Bayesian mixed models with Markov Chain Monte Carlo, correcting for phylogenetic relatedness, using the R package MCMCglmm (Hadfield, 2010). We controlled for chain convergence visually and with the Heidelberg stationarity test diagnostic function of convergence heidel.diag from the R package coda (Plummer et al., 2006).

To get accurate estimations of posterior distributions and avoid chain autocorrelation, we adjusted the iterations, burn-in and thinning for each model to ensure an effective sample size across all parameters of at least 1000. Models were run with a weakly informative prior for random effect and residual variances ($V = 1$, $\nu = 0.002$) not to constrain the exploration of parameter values. We selected the best model with a backward selection of fixed parameters based on Bayesian p-value of 0.05, within the 95% credibility interval excluding zero. In all the

models, species identity was implemented as a random factor. When the dataset was based on the average measurements of the most common species (i. e., all analyses involving buffering), we performed Phylogenetic Generalized Least Squares models (PGLS) using the package *caper* in R (Orme et al., 2013), which allows one value per species.

3. Results

Highland and lowland habitats clearly have distinct temperature profiles, both in their average or extreme temperatures as well as temperature range, confirming they can potentially exert different pressures on species (Table 1).

Butterfly activity and buffering

Species activity was significantly dependent on the community it originated from (Pearson's chi square test, $\chi^2=32.004$, $df=3$, $p\text{-value}<0.001$). A higher proportion of individuals of the lowland community were flying while collected, with very few individuals inactive. We recorded no butterfly basking in any of the collecting sites, regardless of the weather (Fig S1).

We found that the flight conditions for these two communities, i.e. ambient temperature, were significantly different (Welsh two samples t-test: $t = -65.363$, $df = 682.91$, $p\text{-value} < 0.001$). The average ambient temperature for which we recorded individuals flying was 18.13°C in highlands, and 25.99°C in lowlands (Fig S2). These temperatures are slightly above the usual average temperatures for these sites (Table 1), likely because butterflies can only be collected during dry days, which are on average warmer than rainy days.

We compared the buffering ability of species and communities by calculating the slope of body temperature against air temperature of the most common species (Table S2, Fig S3). Whether in the linear model or with the PGLS model correcting for phylogeny, we found that species from the highland community had a higher slope than species of the lowland community. Highland species had and on average a slope >1 , while the average slope in the lowland community was <1 (Table S3, S4, Fig 1). There was no effect of butterfly dry weight on the slope.

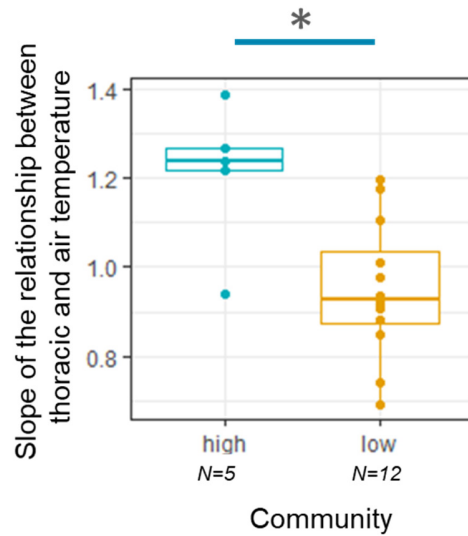


Figure 1. Slope of the thoracic temperature against air temperature of the most common species according to their communities. The significance of the difference between the two groups is based on the PGLS model correcting for phylogenetic relatedness.

Thermal Tolerance to cold and heat stress

Highland butterflies were more resistant to a 5°C cold stress than their lowland counterparts. The time until they fell into a knock-out state was significantly longer, as proven by the mixed models and MCMCglmm models correcting for phylogeny (Table S5,S6, Fig 2, Fig S4). Individuals with a higher weight resisted longer, an effect that was not held in the model correcting for phylogeny, as it is related to phylogeny. By contrast, no significant difference was found regarding the recovery capacity after the cold shock trial (Table S7,S8, Fig 2, Fig S4).

Highland butterflies were less resistant to heat shock at 39°C than lowland butterflies, as their time until knock out was significantly lower than that of lowland individuals according to the linear mixed model and the MCMCglmm models correcting for phylogeny (Table S9,S10, Fig 2, Fig S4). There was no effect of butterfly dry weight. Sex had no effect in either of the tolerance tests.

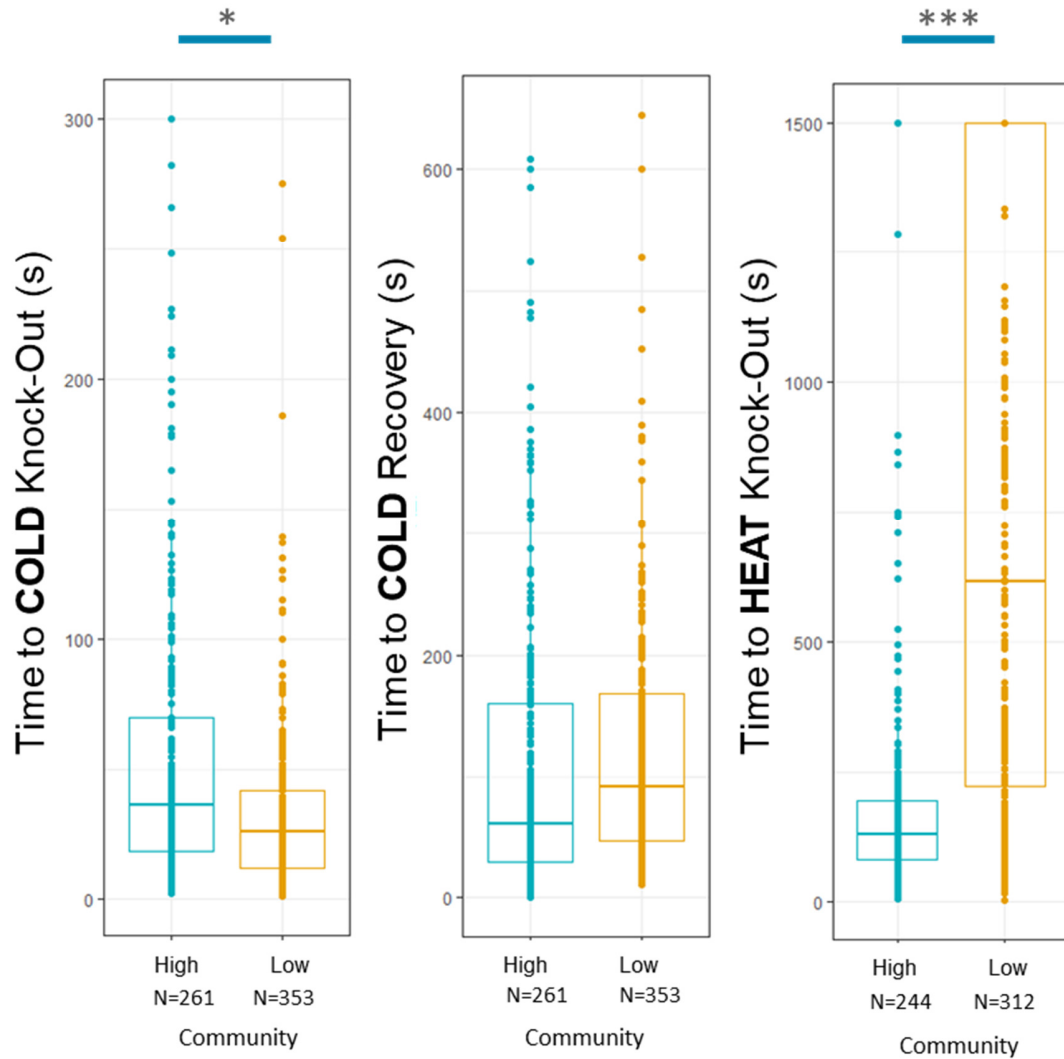


Figure 2. Thermal tolerance comparison between the highland and lowland communities of butterflies. From left to right: Time until knock-out in seconds for the highland and lowland communities during the cold shock experiment; time until recovery in seconds for the highland and lowland communities during the cold shock experiment (5 °C); and time until knock-out in seconds for the highland and lowland communities during the heat shock experiment (39 °C). Each dot represents the mean knock-out time of a species. The significance of the difference between the two groups is based on the Bayesian p-value of the mixed models corrected for phylogeny.

4. Discussion

In this study we investigated the response to temperature across an altitudinal gradient in ithomiine butterflies. We found differences in buffering abilities and cold and heat tolerance between lowland and highland species.

Difference in buffering abilities between communities

Differences in the relationship between thoracic and air temperature were found between species in communities of different elevations. Slopes of the lowland species are closer to 0 than at higher altitude, indicating little need for thermal buffering. At high elevations, the slopes were surprisingly greater than 1 for three out of five species, and no slope is less than 1. Although not predicted by theory, slopes greater than 1 have been reported in the literature: in *Calopteryx* damselflies, authors interpret such slope values as an indication of a thermoregulatory ability: raising body temperature more as ambient temperature increases could be a rapid way of reaching an internal temperature closer to the metabolic optimum, thus avoiding prolonged suboptimal functioning (Schreiner et al., 2020). Yet, such values have also been interpreted as a lower thermal efficiency, indicating that the effectiveness at thermoregulating decreases when the thermal quality of the environment decreases, as observed in butterfly imagoes (Bladon et al., 2020), larvae (Ashe-Jepson et al., 2023b), and lizards (Nordberg and Schwarzkopf, 2019); or as neutral, such as in tortoise, whose buffering strategy varies with the time of day (Hailey and Coulson, 1996). In a study comparing buffering capacity between tropical and temperate communities, but without applying a phylogenetic correction, Laird-Hopkins et al. (2023) also shows results that are counter-intuitive, with tropical species thermoregulating more than temperate ones. They show that, more than climate, morphology is the factor that most influences thoracic temperature, which was not the case in our study.

Our results indicate that highland ithomiine species are able to cope with the ambient conditions and maintain activity without a clear buffering need. Therefore, thermal requirements are most likely different between the communities. To refine the exploration of thermal needs, other tests are necessary to understand on a fine scale the optimum temperature conditions for these species to be able to fly (Nève and Hall, 2016). In addition, the actual temperature felt by an individual, or operative temperature, is not only related to ambient temperature, but also to other environmental factors, such as solar radiation and air movement (Dzialowski, 2005), which can influence individual regulation responses.

Thermal tolerances as a response to temperature variation along the Andean altitudinal gradient

We found a clear evidence for a divergent response towards extreme climatic stress conditions between the two communities. Low-altitude species rapidly drop to a knock-out state when exposed to a temperature of 5°C, while, on average, they resist for a long time to a temperature as high as 39°C. By contrast, species from the highland community, which show better resistance to cold, are much more sensitive to exposure to extreme heat, in line with the prediction, and the trend described for the populations of *Heliconius* butterflies (Montejo-Kovacevich et al., 2020). Similarly, the ability of Colombian butterfly communities to recover from a cold shock is directly linked to climatic conditions, but with a strong effect of morphology (Khazan et al., 2022). In our study, heavier individuals seem to better withstand cold stress, as they have a greater thermal inertia (Porter and Gates, 1969), but this result is not persistent when we take phylogenetic relatedness into account, probably because morphology is closely linked to phylogeny. Intriguingly, while recovery after a cold stress is a cue widely used to assess thermal tolerance in ectotherms, because it can predict individuals' ability to get active in the morning hours, we found no significant difference in the recovery performances of the two communities in our cold assays, despite a trend in the expected direction (i. e., a quicker recovery in highland butterflies).

No clear behavioural response to temperature variation

Our results suggest that it is unlikely that behaviour, such as orienting body or wings towards the sun, plays a major role in thermoregulation, as no basking behaviour has been observed. Yet, we would need a proper observation protocol continuously throughout the day to confirm this preliminary observation. Basking is likely more difficult to detect, especially in clearwing butterflies. Yet it is expected that at high altitudes, ectotherms spend more time trying to warm up and expose themselves to the sun during the day than at low altitudes (Lambrinos and Kleier, 2003). In butterflies, microhabitat preference may play a crucial role to buffer body temperature, as is the case in a temperate community of species from the genus *Erebia* (Nymphalidae): while different species share the same general habitat in the Alps, they are distinguished by their active choice of diverging microhabitat for protection or exposure to heat (Kleckova et al., 2014). In what appears to be a homogeneous environment, the vegetation provides a number of different temperature patches offering a mean to alleviate the effect of global temperature (Montejo-Kovacevich et al., 2020; Pincebourde and Suppo, 2016). Still, high altitude species were caught flying at significantly lower temperatures, associated with lower thoracic temperatures in general. This result shows that there is a local response to climate, as confirmed by the differences between communities in the knockdown assays.

Environmental filtering, adaptation and plasticity

There are several types of response to selection pressures: environmental filtering, where an environment cannot be colonized by species devoid of specific traits that enable them to thrive in this environment; genetic adaptation, when traits of species inhabiting a given environment have evolved under selection from that environment; and plasticity, when individuals express traits at a level that depends on the conditions they are exposed to, and this expression can thus vary with space and time. Filtering typically takes the form of phylogenetic clustering, under the assumption that traits are conserved (Webb et al., 2002). In other words, communities that were assembled through environmental filtering consist of species that are more closely related than if communities consisted of random samples of species from the region. Under such phylogenetic clustering, if a relationship is detected by standard models between two traits (e. g., a thermal-related trait and an altitudinal niche), this relationship will likely not hold in analyses controlling for the phylogeny. By contrast, if a relationship is detected even in phylogeny-controlled analyses, this means that genetic adaptation or plasticity must be at play. Several studies on the adaptation of ectotherms to temperature have highlighted the joint responsibility of genetic and plastic effects, whether in terms of morphology, coloration or thermal tolerance (Fischer and Karl, 2010; Markl et al., 2022).

In Ithomiini, there is probably some filtering by altitude, as suggested by the phylogenetic clustering along the Andean altitudinal gradient, and all the more as the extent of clustering increases with elevation (Chazot et al., 2014). Indeed, while highland and lowland communities have similar species richness, highland communities are dominated by three genera, whereas lowland communities contain more diverse lineages (Chazot et al., 2014). Despite such filtering by altitude, our results controlling for phylogeny indicate an altitudinal effect on ithomiine thermal tolerances independent of the phylogeny, suggesting that filtering is not the only process to act, and that thermal tolerances either have evolved *in situ* under natural selection imposed by local climatic conditions, or are the result of adaptive plasticity.

The study of the thermal tolerance of *Heliconius* butterfly (Nymphalidae) low and mid-altitude populations shows that, at the intraspecific level, there is both adaptation and plasticity (Montejo-Kovacevich et al., 2020). By contrast, our study focuses on the interspecific scale, and none of the species is found at both altitudes. Moreover, some entire lineages are restricted to specific altitudes (Chazot et al., 2014). For instance, the genera *Patricia*, *Veladyris* and *Velamysta* only occur at high altitudes, while *Tithorea*, *Pseudoscada* and *Heterosais* are found at low altitudes. Our experiments do not allow to distinguish between plasticity and genetic adaptation. The extent of thermally-induced plasticity in Ithomiini remains to be investigated.

Conclusion

Ithomiini are unusual butterflies since most of the species in this group have transparent or partially transparent wings (Pinna et al., 2021). Although detrimental to the absorption of light and heat (Ossola et al., 2023), these clearwing species are nevertheless found at very high altitudes (Doré et al., 2022) and the high-altitude community is composed exclusively of transparent species, against all expectations (Chazot et al., 2014; Ossola et al., 2023). Ithomiine wing coloration therefore does not seem to contribute to adaptation to altitude, which departs from studies reporting a correlation between darkness and climate in butterflies (Kang et al., 2021; Munro et al., 2019). Here, we found no clear evidence for behavioural temperature adjustment, and we revealed unusual buffering patterns that could be interpreted either as a poor thermoregulatory ability or a potential boost in thermoregulation at high temperatures in highland species. Yet, our results provide support for the existence of physiological adaptation to different temperature conditions in Ithomiini, be it genetic or plastic. Our results raise concerns about the impact of climate change on populations of Ithomiini, and more generally of tropical insects with narrow altitudinal niches (Perez et al., 2016). The fact that we did not detect buffering ability or behavioral adjustment put the species facing unexpected temperature variations at risk, with little possibility to tune their response.

Aknowledgments

This work was funded by Clearwing ANR project (ANR-16-CE02-0012), HFSP project on transparency (RGP0014/2016). VO is recipient of a doctoral fellowship from the doctoral school FIRE, Learning Planet Institute. We thank Carlos Robalino, Maria-José Sanchez, Guillaume Ghisbain, Raul Aldas, Ismael Aldas, for the help in the field and with the experiments. We thank Caroline Baquet, Nicolas Nadeau, Keith Willmott and Mathieu de Goulaine for their expertise on butterflies, tropical systems, and assistance in setting up the study. We thank Sofía Nogales for granting export permits.

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Chapter 2 – Appendix

Supplementary Figures and Tables

Table S1. List of the species and the number of specimens per species collected, and the community they belong to.

Species	Community	Number of individuals collected
<i>Aeria eurimedia</i>	Low altitude	1
<i>Brevioleria arzalia</i>	Low altitude	8
<i>Callithomia lenea</i>	Low altitude	2
<i>Ceratinia poecila</i>	Low altitude	32
<i>Dircenna dero</i>	Low altitude	1
<i>Dircenna loreta</i>	Low altitude	1
<i>Episcada sulphurea</i>	Low altitude	1
<i>Forbestra equicola</i>	Low altitude	1
<i>Forbestra proceris</i>	Low altitude	1
<i>Godyris panthyale</i>	High altitude	1
<i>Godyris zavaleta</i>	Low altitude	20
<i>Heterosais giulia</i>	Low altitude	1
<i>Heterosais nephele</i>	Low altitude	6
<i>Hypoleria alema</i>	Low altitude	8
<i>Hypomenitis enigma</i>	High altitude	1
<i>Hypomenitis hermana</i>	High altitude	2
<i>Hypomenitis lydia</i>	High altitude	23
<i>Hypomenitis oneidodes</i>	High altitude	3
<i>Hypomenitis ortygia</i>	High altitude	2
<i>Hypomenitis theudelinda</i>	High altitude	2
<i>Hyposcada anchiala</i>	Low altitude	27
<i>Hyposcada illinissa</i>	Low altitude	10
<i>Hypothyris anastasia</i>	Low altitude	5
<i>Hypothyris euclea</i>	Low altitude	11
<i>Hypothyris moebiusi</i>	Low altitude	4
<i>Hypothyris ninonia</i>	Low altitude	1
<i>Ithomia agnosia</i>	Low altitude	2
<i>Ithomia avella</i>	High altitude	2
<i>Ithomia cleora</i>	Low altitude	3
<i>Ithomia salapia</i>	Low altitude	85
<i>Mcclungia cymo</i>	Low altitude	3
<i>Mechanitis lysimnia</i>	Low altitude	14

Species	Community	Number of individuals collected
<i>Mechanitis mazaesus</i>	Low altitude	1
<i>Mechanitis messenoides</i>	Low altitude	7
<i>Mechanitis polymnia</i>	Low altitude	22
<i>Mechanitis sp</i>	Low altitude	1
<i>Megoleria orestilla</i>	High altitude	5
<i>Melinaea idae</i>	Low altitude	2
<i>Melinaea mothone</i>	Low altitude	1
<i>Methona confusa</i>	Low altitude	34
<i>Methona curvifascia</i>	Low altitude	1
<i>Napeogenes inachia</i>	Low altitude	5
<i>Napeogenes larilla</i>	High altitude	1
<i>Oleria assimilis</i>	Low altitude	2
<i>Oleria baizana</i>	High altitude	139
<i>Oleria cyrene</i>	High altitude	56
<i>Oleria gunilla</i>	Low altitude	7
<i>Oleria makrena</i>	High altitude	5
<i>Oleria onega</i>	Low altitude	45
<i>Oleria tigilla</i>	Low altitude	38
<i>Oleria tremona</i>	High altitude	88
<i>Oleria zelica</i>	Low altitude	5
<i>Patricia dercyllidas</i>	High altitude	2
<i>Pseudoscada florula</i>	Low altitude	3
<i>Pseudoscada timna</i>	Low altitude	9
<i>Pteronymia alina</i>	High altitude	1
<i>Pteronymia ticida</i>	High altitude	3
<i>Pteronymia veia</i>	High altitude	7
<i>Pteronymia vestilla</i>	Low altitude	10
<i>Scada reckia</i>	Low altitude	2
<i>Thyridia psidii</i>	Low altitude	1
<i>Tithorea harmonia</i>	Low altitude	2
<i>Veladyris pardalis</i>	High altitude	88
<i>Velamysta pupilla</i>	High altitude	3

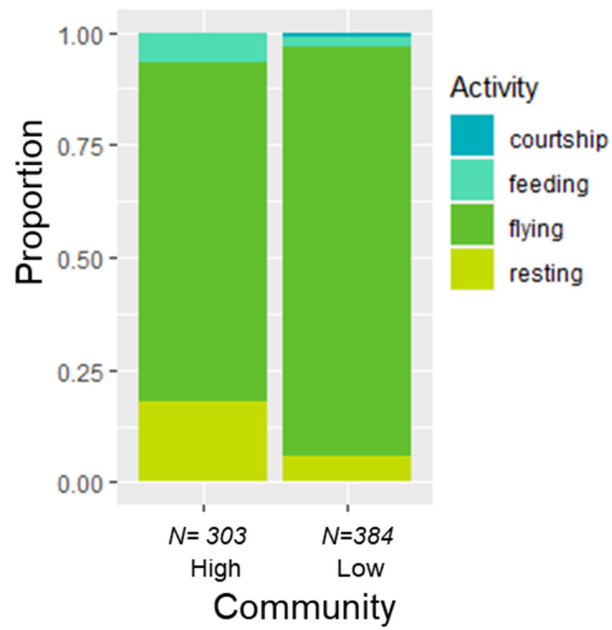


Figure S1. Comparison of the proportion of individuals and their type of activity of the two communities of butterflies. No basking behaviour was observed (N high = 434, N low = 458).

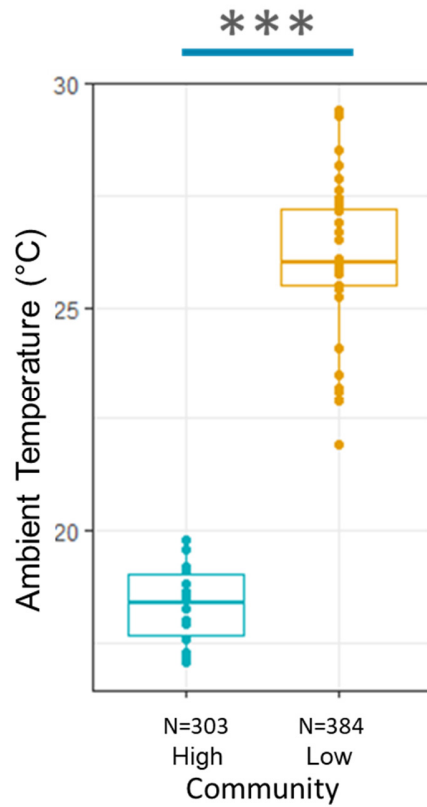


Figure S2. Comparison between the mean ambient temperature at which highland and lowland individuals were recorded flying. Each dot represents the mean ambient temperature for each species. The significance of the difference between the two groups is based on the Welsh two samples t-test, with p-value set at 0.05.

Table S2. Slope parameters of the thoracic temperature in function of the air temperature of the most common species (>10 individuals), extracted from a linear regression, with a confidence interval set at 0.95.

Species	Community	N observations	intercept	slope	CI inferior	CI superior
<i>Godyris zavaleta</i>	low	20	0.917	1.011	0.840	1.182
<i>Mechanitis polymnia</i>	low	22	-3.323	1.177	0.997	1.357
<i>Hyposcada illinissa</i>	low	10	4.437	0.881	0.693	1.069
<i>Hypothyris euclea</i>	low	11	-4.151	1.197	0.663	1.731
<i>Ithomia salapia</i>	low	85	3.901	0.906	0.785	1.026
<i>Oleria tigilla</i>	low	38	4.835	0.849	0.612	1.085
<i>Ceratinia poecila</i>	low	32	-0.915	1.104	0.823	1.385
<i>Mechanitis lysimnia</i>	low	14	3.917	0.919	0.552	1.286
<i>Pteronymia vestilla</i>	low	10	9.558	0.693	0.206	1.180
<i>Methona confusa</i>	low	34	3.322	0.936	0.635	1.238
<i>Hyposcada anchiala</i>	low	27	8.183	0.740	0.311	1.169
<i>Oleria onega</i>	low	45	1.739	0.976	0.772	1.180
<i>Oleria tremona</i>	high	88	-1.898	1.215	1.046	1.384
<i>Oleria baizana</i>	high	139	-2.562	1.238	1.049	1.428
<i>Hypomenitis lydia</i>	high	23	-5.382	1.388	0.777	1.999
<i>Veladyris pardalis</i>	high	88	2.607	0.939	0.723	1.156
<i>Oleria cyrene</i>	high	56	-2.833	1.267	1.005	1.528

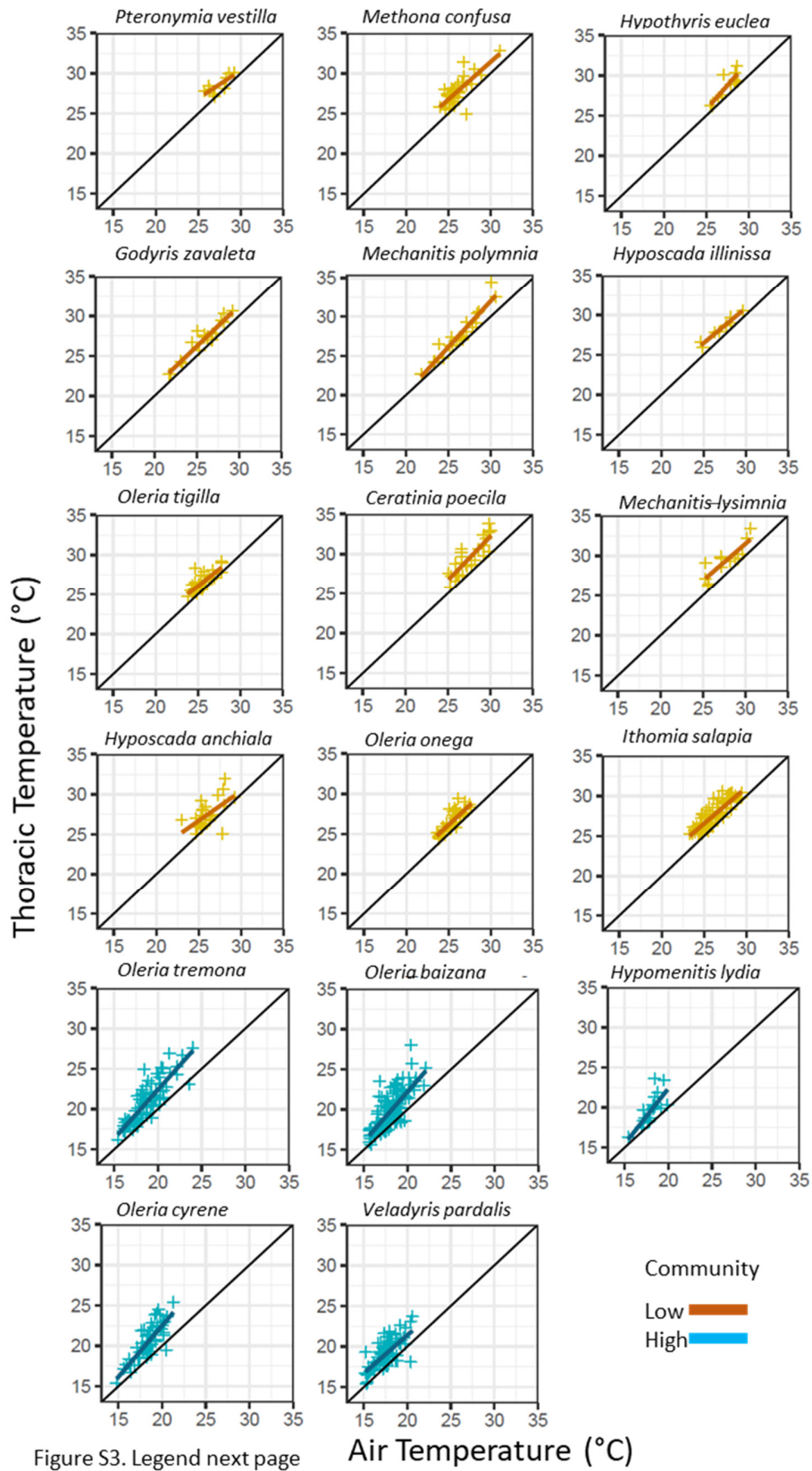


Figure S3. Legend next page

Figure S3. Thoracic temperature of the most common species sampled against the ambient air temperature. Points represent individuals. The species are categorised according to the community: highland in blue or lowland in yellow. The slope and number of individuals for each species is given in Table S2).

Table S3. Retained linear model of the relationship between the slope of the thoracic temperature against air temperature of the most common species, and the community and average weight of the species. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	Value	Std.Error	T.value	p-value
Slope (Tthorax ~T air)	(Intercept)	1.209	0.071	17.041	<.001***
	Community High>Low	-0.260	0.084	-3.083	<.001***
	Factor not retained : weight				

Table S4. Results of the PGLS model, correcting for phylogenetic relatedness, ($F_{2,14} = 4.43$ (p-value <0.05*), $Radj^2 = 0.3006$, $\lambda = 0$ (probability($\lambda=1$) < 0.001)), of the link between the slope of the thoracic temperature against air temperature of the most common species in function of their communities, after backward selection of fixed parameters based on p-value criteria. The factors for which the parameters were not significant are listed below the model. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	Estimate	Std. Error	t value	Pr(> t)
Slope (Tthorax ~T air)	(Intercept)	1.206	0.096	12.5123	<0.001**
	Community High>Low	-0.261	0.089	-2.9498	<0.05*
	Factor not retained : weight				

Table S5. Retained linear mixed model of the relationship between time until knock-out of the butterflies during the cold shock experiment (5°C) and their community, after backward selection of fixed parameters based on the p-value. The factor for which the parameters were not significant is listed below the model. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	Value	Std.Error	DF	T.value	Estimated std Error	p-value
Time to COLD Knock-Out	(Intercept)	-23.998	15.912	518	-1.508	-24 ± 15.91	0.132
	Community High>Low	29.922	5.874	54	5.094	29.92 ± 5.87	<.001***
	Weight	0.028	0.012	518	2.429	0.03 ± 0.01	0.015*
	SD of the temperature in the jar	47.044	9.765	518	4.817	47.04 ± 9.77	<.001***
	Mean temperature in the jar	11.469	2.801	518	4.095	11.47 ± 2.8	<.001***
Other fixed parameters not retained : sex							

Table S6. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between time until knock-out of the butterflies during the cold shock experiment (5°C) and their community, after backward selection of fixed parameters based on Bayesian p-value criteria.

Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian p-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian p-value
Time to COLD Knock-Out	(Intercept)	-4.139	-40.109	31.789	64370.475	0.812
	Community High>Low	-35.508	-51.267	-20.491	24963.503	< 0.001 ***
	Mean temperature in the jar	11.540	6.279	16.920	59416.993	< 0.001 ***
	SD of the temperature in the jar	44.068	25.011	62.724	67253.280	< 0.001 ***
	phylogenetic variance	374.808	77.171	755.170	2512.593	
	Species identity	9.866	0.000	57.054	1762.118	
	residual variance	1995.147	1758.573	2235.284	61902.755	
Other fixed parameters not retained : weight; sex						

Table S7. Retained linear mixed model of the relationship between time until recovery of the butterflies during the cold shock experiment (5°C) and their community, after backward selection of fixed parameters based on the p-value. The factor for which the parameters were not significant is listed below the model. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	Value	Std.Error	DF	T.value	Estimated std Error	p-value
Time to COLD Recovery	(Intercept)	132.444	51.035	514	2.595	132.44 ± 51.04	0.01**
Fixed parameters not retained : Community, weight, sex, mean temperature in the jar, standard deviation of the temperature in the jar, temperature difference between the ambient temperature experienced by the butterfly during recovery and the average temperature experienced by butterflies during recovery of the same community							

Table S8. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between time until recovery of the butterflies during the cold shock experiment (5°C) and their community, after backward selection of fixed parameters based on Bayesian p-value criteria. (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian p-value
Time to COLD Recovery	(Intercept)	126.803	101.904	148.875	7658.825	<.001***
	phylogenetic variance	248.851	0.000	1488.694	564.340	
	Species identity	627.911	0.000	1770.362	1451.099	
	residual variance	21341.327	18796.220	23879.749	37589.352	
Fixed parameters not retained : community; weight; sex; Mean temperature in the jar; standard deviation of the temperature in the jar; temperature difference between the ambient temperature experienced by the butterfly during recovery and the average temperature experienced by butterflies during recovery of the same community						

Table S9. Retained linear mixed model of the relationship between time until knock-out of the butterflies during the heat shock experiment (39°C) and their community after backward selection of fixed parameters based on the p-value. The factor for which the parameters were not significant is listed below the model. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	Value	Std.Error	DF	T.value	Estimated std Error	p-value
Time to HEAT Knock-Out	(Intercept)	4157.340	1413.179	515	2.942	4157.34 ± 1413.18	<.01**
	Community Low>High Mean	639.372	82.646	54	7.736	639.37 ± 82.65	<.001***
	temperature in the jar	-101.466	36.174	515	-2.805	-101.47 ± 36.17	<.01**
	Other fixed parameters not retained : weight; sex; standard deviation of the temperature in the jar						

Table S10. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between time until knock-out of the butterflies during the heat shock experiment (39°C) and their community, after backward selection of fixed parameters based on Bayesian p-value criteria.

Fixed effect estimates with 95% credibility intervals excluding zero are indicated in bold, associated with a Bayesian p-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % credibility Interval	Upper 95 % credibility Interval	Effective size	Bayesian p-value
Time to HEAT Knock-Out	(Intercept)	5287.928	2146.456	8428.301	59000.00	< .001 ***
	Community Low>High Mean	549.201	366.836	729.861	52516.453	< .001 ***
	temperature in the jar	-125.583	-205.986	-45.712	59000.00	< .01 **
	phylogenetic variance	73086.650	19117.370	145187.687	2102.899	
	Species identity	1436.552	0.000	8598.810	1400.351	
	residual variance	161936.321	141083.900	182679.897	37537.672	
Other fixed parameters not retained : weight; sex; standard deviation of the temperature in the jar						

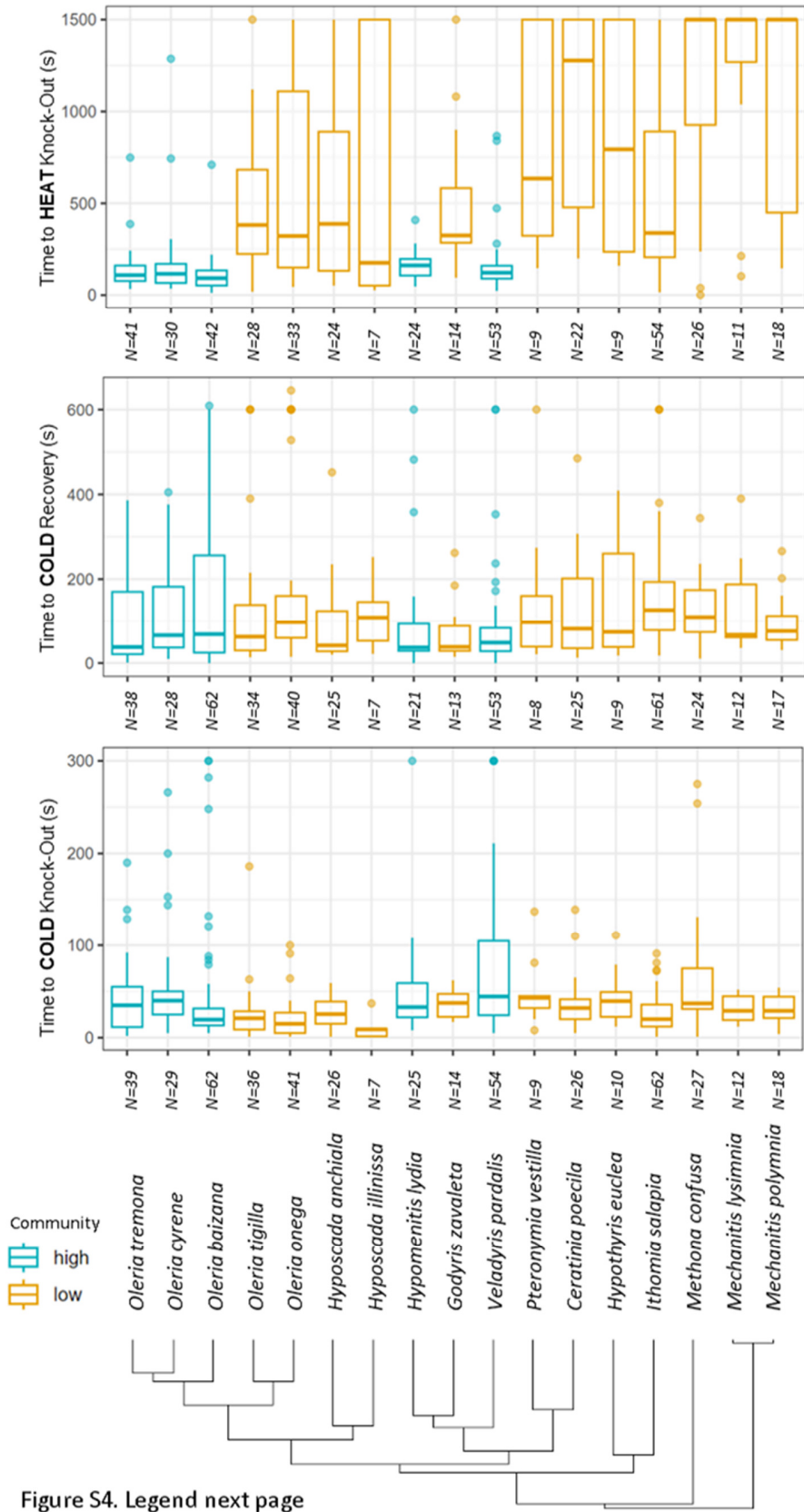


Figure S4. Thermal tolerance comparison between the most common species sampled among the highland and lowland communities of butterflies, reordered according to their phylogenetic relationships. From bottom to top : Time until knock-out in seconds of the most common species sampled among the highland and lowland communities during the cold shock experiment; time until recovery in seconds of the most common species sampled among the highland and lowland communities during the cold shock experiment (5°C); and time until knock-out in seconds of the most common species sampled among the highland and lowland communities during the heat shock experiment (39 °C).

Chapter 3. Testing for thermally induced plasticity of wing traits and thermal tolerance in two species of *Ithomiini*

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Abstract

Temperature has a long-documented effect on ectotherm development, and has the potential to induce plastic effect on their morphology and thermal performances. In butterflies, the effect of developmental temperature can influence wing traits linked to heat absorption capacities, such as the degree of melanization. Here, we study the effect of three temperature treatments on the developmental and trait plasticity of two species of Panamanian Ithomiini butterflies (Nymphalidae), *Methona confusa* and *Tithorea harmonia*. We measured developmental parameters, such as development time, survival and body weight of larvae, and the weight of imagoes, reared at a constant temperature of either 18°C, 24°C or 27°C. We tested the effect of the treatments on thermal tolerance to cold and heat stress of the butterflies, and compared them to that of individuals from the wild populations. Larval development was significantly influenced by temperature: in warmer conditions, the probability of survival was lower, the development time was shorter, leading to smaller fifth instars and imagoes.

Wing optical properties and degree of darkness were not significantly different among the butterflies exposed to temperature treatment, nor with the wild population. Similarly, the transparent patches on the wings of *M. confusa* were not affected by the rearing temperature. The thermal tolerance to cold stress of *M. confusa* showed thermally induced plasticity. The individuals reared at the 18°C treatment showed the highest cold tolerance, as they took more time to fall in a knock out state and recovered quicker. *T. harmonia* cold resistance did not show significant differences between treatments, but wild individuals were more resistant to cold knock-out. Heat stress tolerance was not significantly influenced by the developmental temperature in either species, as we did not detect any significant difference between treatments. In summary, developmental temperature in these two species of butterflies has a strong effect on immature stages, but no effect on the wing properties linked to temperature absorption. Moreover, our results suggest that the resistance to cold is at least partly due to plasticity in these species. The absence of variation regarding heat resistance and the high mortality of larvae at the high temperature treatment in both the species raises questions on the impact of climate change on tropical species.

1. Introduction

Species distribution is limited by environmental factors, especially in ectotherms. Their body temperature is directly linked to their environment, which incurs selection on various morphological and physical traits, such as body coloration (Clusella Trullas et al., 2007), body mass (Atkinson, 1994), or tolerance to thermal stress (Sunday et al., 2011). Temperature resistance and thermal limits of species for a given physiological performance vary according to climatic conditions, and is therefore directly related to the latitude or altitude at which the organism lives (Sunday et al., 2011; García-Robledo et al., 2016; Polato et al., 2018). For instance, in tropical Andean butterflies, tolerance to cold and heat stress varies according to altitude: cool-climate taxa at high altitudes are more resistant to cold but more sensitive to heat than low-altitude taxa (chapter 2; Montejo-Kovacevich et al., 2020).

In insects, during the immature stages, temperatures can have a major influence on development and survival (Huang et al., 2022, Kingsolver and Huey, 2008). The rate of development follows a temperature-performance curve: a linear trend with temperature is observed, with a maximal upper temperature beyond which the accumulation of damages is too important and the development rate drops. Thermal stress often results in reduced fitness, high temperature has a negative effect on survival probability (Huang et al., 2022) along with a negative impact on the reproductive success (Kingsolver and Huey, 2008; Awde et al., 2023).

Multiple mechanisms enable ectotherms to maintain an adequate temperature and to cope with difficult climatic conditions. Physical factors such as melanization and body size play a role in heat absorption and insulation (Watt, 1968; Bishop et al., 2016). Underlying physiological characteristics such as heat shock proteins or cryoprotectant help alleviate the effects of thermal stress in extreme climates (Vrba et al., 2017). In addition, because fine temperature variations can have a direct impact on their activity, ectotherms rely on various behavioral responses to buffer their temperature, such as tuning solar radiation intake via body orientation, shivering, selecting microhabitat and modulating their activity according to the time of the day (Heinrich and Pantle, 1975; Lambrinos and Kleier, 2003; Verdú et al., 2012; Barton et al., 2014; Kleckova et al., 2014). However, because of its unprecedented pace, the current climatic change, with rising temperatures and more frequent extreme climatic events, is highly challenging for ectotherms, which are particularly at risk of being set away from a range of conditions they can withstand.

Many studies over the last 25 years have shown that insect species tend to track their niche by shifting their distribution to cooler environments, in altitude or towards the poles, since a first description of this phenomenon in the North American butterfly *Euphydryas editha* (Nymphalidae) (Parmesan et al., 1999). Such distribution shifts highly threaten species that have already reached their maximum upper altitudinal range (Halsch et al., 2021). However, 85% of these studies focus on European and North American taxa (McCain and Garfinkel, 2021). Consequently, the effect of climate changes on tropical communities is still poorly understood. Yet, climate change could be even more devastating for tropical than for

temperate species. Tropical species are usually exposed to a narrow range of environmental conditions and to a much less fluctuating climate throughout the year than in temperate zones, and climate change exposes them to conditions they have never experienced before (Williams et al., 2007).

The phenotypic responses of thermal-linked traits to variation of the environmental conditions in a population may stem from genetic variation and/or environmentally induced phenotypic plasticity. Much of the inter-individual phenotypic variation in mature individuals comes from the conditions experienced during early stages of development. This period is often critical and can have persistent effects on the physiology and metabolism of adults (Eyck et al., 2019; O'Dea et al., 2019), and the induced changes do not necessarily have a genetic basis and can be reversible (Charmantier et al., 2008). It is difficult to distinguish the exact contribution of genetic and plastic responses, but many studies acknowledge that the two types of response likely act jointly to explain the observed traits (Fischer and Karl, 2010; Montejo-Kovacevich et al., 2020). Developmental plasticity is a specific case of plastic response. Many traits linked to thermal adaptation in adult butterflies have been shown to vary in response to temperature conditions experienced in early stages. For instance, the surface of melanic areas (i. e., dark patches) on the wings is usually larger in individuals that have experienced colder conditions in populations (e. g., Davis et al., 2005; Otaki, 2008; Markl et al., 2022). Tolerance to temperature stress and upper and lower thermal limits in adult ectotherms are also often linked to conditions experienced in immature stages e.g. (Fischer and Karl, 2010; Eyck et al., 2019; Montejo-Kovacevich et al., 2020; Awde et al., 2023). Phenotypic plasticity, however, is not always adaptive (Merilä and Hendry, 2014), and can have contrasting effects on fitness. For instance, warm developmental condition in insects results in quicker development rate and smaller imagoes. Although this enables them to reach sexual maturity sooner, it is associated with lower reproductive success (Kingsolver and Huey, 2008). Moreover, phenotypic changes on thermal tolerance during acclimation are sometimes accompanied by deleterious effects on adult fertility, as shown in *Drosophila* sp. (Ayrinhac et al., 2004). Understanding plasticity as a response to temperature variations in tropical insects is essential to anticipate the effects of climate change on tropical insects.

In Ithomiini (Nymphalidae), a clade of Neotropical butterflies found from the lowlands up to 3000 meters of altitude in the Andean mountains (Chazot et al., 2019; Doré et al., 2022), wing coloration does not seem to be part of thermal adaptation. In Ithomiini, nearly 80% of the 396 species harbor partially transparent wings, a feature that incurs a thermal cost (Ossola et al., 2023). Unexpectedly, the proportion of transparent species and the extent of transparency increases with altitude (Ossola et al., 2023), in sharp contrast with the main patterns of climate-dependent insect coloration described in the literature (Zeuss et al., 2014; Munro et al., 2019; Kang et al., 2021). As such, Ithomiini challenge the hypotheses of thermal melanism, which posits that colder environments is expected be home to darker-colored individuals and species (Clusella Trullas et al., 2007), thereby enabling better heat absorption. While the behavior of Ithomiini does not seem to play a role in the thermoregulation of these

butterflies either (chapter 2), we showed that the thermal capacities of high- and low-altitude communities are different, and that thermal tolerances at temperature extremes (hot and cold) are also different (chapter 2). Specifically, ithomiine species living at high altitudes are more resistant to cold but less resistant to heat than species living in the hot lowlands, even when accounting for shared ancestry. However, because those results were obtained using wild-caught individuals, it is unknown whether the differences stem from genetic differences or from thermally-induced plasticity. Test of heat tolerance on other species of Andean butterflies belonging to the nymphalid tribe Heliconiini showed that part of the response to hot temperature could be attributed to plasticity (Montejo-Kovacevich et al., 2020).

The aim of the current study is to use an experimental setting to assess the impact of different development temperatures on immature (egg, larva and pupa) survival and growth, and on the extent of plasticity in thermal responses of ithomiine species. We reared two ithomiine species from Panama with contrasting extent of transparency, the clearwing *Methona confusa* and the opaque *Tithorea harmonia* (Fig 1), from egg to imago, at three temperature treatments: 18, 24 and 27 °C.

First, we tested whether temperature affects development and survival at immature stages. Warmer temperatures are expected to induce quicker development, but smaller larval weight. In addition, we predicted a lower survival rate for the warmest treatment, because it is warmer than the average temperature experienced by individuals of the two species in their natural environment. Then, we explored how physical and morphological traits respond to temperature development. Traits measured include adult weight, wing surface occupied by melanic patches and optical absorption properties of the wings over the range 300 to 2500 nm (which comprises the ultraviolet (UV), Visible and near-infrared (NIR) ranges). If traits are subject to thermally induced plasticity, we expect to observe smaller adults in warm conditions, with lighter colorations and a lower proportion of solar radiation absorption, especially in the NIR. Finally, we measured the influence of temperature during development on the thermal response of adult butterflies to heat and cold extremes stress conditions. We expect that individuals reared in warmer conditions should have a lower cold stress tolerance, but a better heat stress tolerance, compared to individuals reared at colder temperatures.

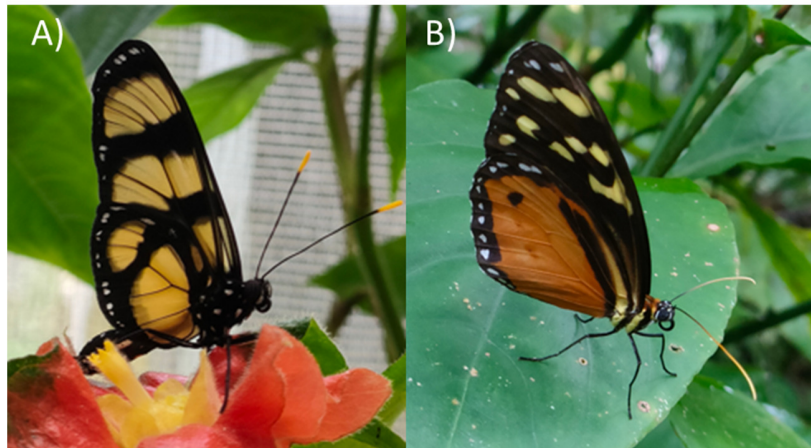


Figure 1. The two ithomiine species studied: A) *Methona confusa* and B) *Tithorea harmonia* (photos: Violaine Ossola).

2. Material and Methods

Rearing protocole

Females of *Tithorea harmonia* and *Methona confusa*¹ were captured in Panama, in wild populations located respectively in Gamboa, Parque Nacional de Soberania, altitude ~ 200 m and in Cerro Azul, altitude ~400 m, in which annual temperature is around 25°C with a breadth of no more than 10°C (Table 1). Females were given an identification number and were put in separate insect cages, in the insectarium of the Smithsonian Tropical Research Institute in Gamboa. Females were offered host plants for them to lay eggs: *Prestonia* sp. for *T. harmonia*, *Brunfelsia* sp. for *M. confusa*.

¹ Initially, a third species, *Greta morgane oto*, was to be included in the protocol. Unfortunately, the death toll among *Greta morgane oto* larvae was so high that we had to remove the species from the study. The proposed explanations for this mortality include fungi or viruses, which are often the cause of high mortality in reared caterpillars. More speculatively, the leaves of *Cestrum* sp., the host plant, may have released toxins once cut from the plant. The last assumption is backed by the same mortality rates in our other attempts to rear *Greta morgane oto* from completely different populations, in a different country, but with the same protocol.

Table 1. Thermal characteristics of the collection sites of the populations from which females and wild individuals tested were collected. The data were extracted from the published WorldClim2 dataset (Fick and Hijmans, 2017), with a resolution of 2.5 arc-minutes based on the sites coordinates.

Collection site	Cerro Azul	Gamboa
longitude	-79.41702	-79.70355
latitude	9.157714	9.121683
Annual Mean Temperature	24.8	26.4
Mean Diurnal Range (Mean of monthly (max temperature - min temperature))	7.6	7.3
Max Temperature of Warmest Month	30.2	31.8
Min Temperature of Coldest Month	20.1	22
Temperature Annual Range (Max Temperature of Warmest Month-Min Temperature of Coldest Month)	10.1	9.8

During two months, the host plants were inspected every day and eggs were removed and carried to the lab for the rearing under controlled conditions. Each batch of eggs laid in one day by a female was randomly split in three equal parts, each of which entered one of three temperature treatments (see below) (Fig 2). We placed the eggs in hermetic transparent plastic cups to avoid desiccation, with host plant leaves provided *ad libitum* and renewed daily. Cups were cleaned every day. Individuals were given a unique identification number and maintained in individual containers all throughout their development until adult stage.

The low, intermediate and high temperature treatments were set at continuous temperatures of 18°C, 24°C and 27°C, respectively, using incubators for the low and high temperature treatments and room controlled temperature for the intermediate treatment, with an artificial light cycle of 12:12 hours. To assess the development rate of immature stages, we monitored the timing (day) of occurrence of the successive developmental stages, named as follows: Laying, hatching, L1 (first larval instar), L2 (second larval instar), L3 (third larval instar), L4 (fourth larval instar), L5 (fifth larval instar), pupation, emergence, imago. Deaths and their timing were also recorded. Eggs of *T. harmonia* were placed in individual cups because of the high propensity for larval cannibalism in this species. By contrast, *M. confusa* larvae are gregarious in the early stages, and larvae were put in individual cups only at L4. L5 larvae were weighted just after moulting, at the precision of 0.001 g. Seven females of *T. harmonia* laid a total of 316 eggs, with 11 (18°C), 22 (24°C), and 2 (27°C) of them surviving through emergence. Nine females of *M. confusa*, laid 990 eggs, with 135 (18°C), 113 (24°C), and 86 (27°C) of them surviving through emergence.

Temperature tolerance tests

Adults that emerged in the morning were tested for cold and heat stress tolerance on the afternoon of the same day. Individuals that emerged later in the day were tested the next day. In addition, wild-caught individuals, 8 *T. harmonia* and 24 *M. confusa*, underwent cold and heat tests. Butterflies were fed with sugary water *ad libitum* one hour before each test to avoid dehydration effects on their performances. To assess the tolerance of the butterflies to temperature stress, we performed cold and heat shock tolerance tests (Ashoff and Schmitt, 2014; Montejo-Kovacevich et al., 2020; Khazan et al., 2022), in the same conditions as in chapter 2.

We first performed a cold shock test, during which the butterfly was placed in a glass jar (320 cm³) with paper at the bottom for it to stand on, along with a thermocouple type K probe to monitor the temperature experienced by the butterfly. The temperature inside the jar was maintained at 5°C throughout the whole test, and was manually adjusted as the jar was placed in or out a container with ice and water as needed. The butterfly was left in the jar for 5 minutes and we monitored the time until we notice knock out, i.e when it was unable to move. After 5 minutes, we took it out of the jar and recorded the time until which the butterfly retrieves the capacity to stand on its legs, noted as the recovery time.

Later in the day, the heat shock trial was performed on individuals that went through a full recovery to cold shock. A water bath Cryothermostat HUBER CC-K20 with a built-in computer controlling the water temperature and homogeneity through water agitation, was equipped with 6 glass jars to test 6 individuals at the same time. Test temperature was set at 39°C. The jars were immersed in hot water, and the opening was covered with mesh. A thermocouple probe inside a jar was used to the temperature throughout the whole trial. We added wet cotton at the bottom of the jar to avoid dehydration. The time until knock out was monitored, and the butterfly was immediately removed from the jar. Butterflies were then frozen and dried to be weighted at the precision of 0.001 g.

Wing thermal trait measurements

To test the effect of developmental temperature on the wing traits of the butterflies, we measured optical properties and degree of darkness of the wing of reared and wild-caught adult butterflies. We took calibrated pictures of one hindwing and one forewing of each individual. We used digital photography to quantify the proportion of dark pigmented surface on the full wings. The background was filled with green, a color that does not occur in wings and that was ignored when calculating the color proportions. We used the `getKMeanColors` function from the `colordistance` R package (Weller and Westneat, 2019) to sort the pixels in clusters according to their RGB values. We set the number of clusters to two, corresponding to mainly dark and brown, versus transparent in *M. confusa* or light yellow and orange colors in *T. harmonia*. We computed the total surface of each wing in mm² in ImageJ 1.52 (Schindelin et al., 2012).

To quantify the extent to which wings transmit and absorb light, we performed spectrophotometry measurements on 90 *M. confusa* and 17 *T. harmonia*, on a hindwing and a forewing for each individual, in the middle of the wing, which falls into a transparent patch for both wings of *M. confusa*, in an orange patch on the hindwing, and a black patch on the forewing of *T. harmonia*.

The measurement was set on a spot of 5 mm of diameter on the hindwing and forewing of the specimens. We measured both total (specular + diffuse) reflection and total (specular + diffuse) transmission spectra of each patch, with an Agilent CARY 5000 UV-Visible-NIR spectrophotometer, equipped with an integrating sphere, over the range 300-2500 nm, which includes ultraviolet (UV), human-visible (Vis) and near-infrared (NIR) wavelengths. Wings were maintained in a black paper mask with an opening of 5 mm of diameter. Spectra were measured relative to a dark (paper mask without the wings, light off) and to a white reference (Spectralon white diffuse reflectance standard), with the paper mask for reflection, nothing for transmission, light on. The resulting total absorption spectra were calculated for each wavelength λ as $\text{Absorption}(\lambda) = 1 - (\text{Transmission}(\lambda) + \text{Reflection}(\lambda))$. The negative values due to the noise of the signal were fixed to zero with the function `fixneg` of the package `Pavo` in R (Maia et al., 2013), and each spectrum was smoothed with the `loess` function in R. We quantified mean absorption at different wavelength ranges, between 300-700 nm, 700-2500 nm, and 300-2500 nm, and mean transmission in the UV-Vis range 300 – 700 nm as a proxy for transparency, as this range is visible by butterflies and their main predators, birds.

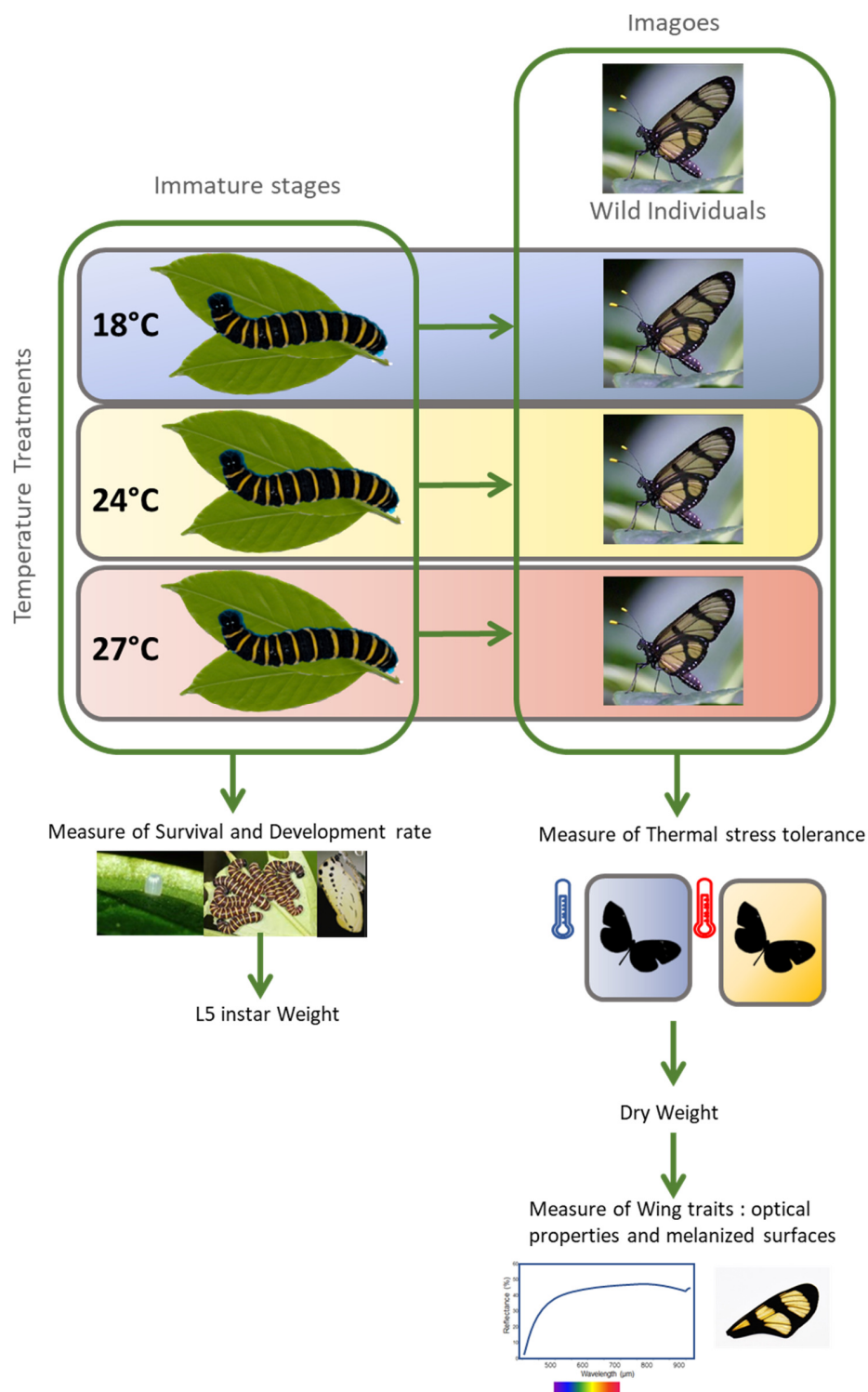


Fig 2. Design of the rearing experiment, with the example of *M. confusa*. The individuals were kept in temperature-controlled chambers from egg to imago. During the larval development, survival, development rate, and the weight of the fifth instar were monitored. Emerged imagoes were taken out of the chamber for the thermal stress assays, after which they were dried to measure the dry weight.

The wing traits were analyzed with spectrophotometry measurement from UV to NIR ranges (300 to 2500 nm), and the proportion of melanized surfaces were calculated from photographs.

Statistical analysis

We examined independently the effect of temperature treatment on the variations in survival rate, development time (days), in L5 instar weight (g), and adult dry weight (g). In adults, we analyzed the effect of temperature treatment on variations in cold knock-out time (s), in cold recovery time (s), as well as heat knock-out time (s). We compared them to those of wild individuals. Finally, wing features were analysed by looking at the effects of temperature on the dark surface proportion of wings, the percentage of absorption of proximal patches in the UV-Visible-NIR range, UV-Visible range and NIR range, and for *M. confusa*, the percentage transmission in the UV-Visible range as a proxy of transparency.

Survival rate during development was tested with a survival model 'coxme', a Cox mixed-effects model fit by maximum likelihood, with the package coxme in R (Therneau, 2020), with mother identity as a random effect, and calendar laying date as fixed effect. Model selection was built on a factor selection based on the z-value (see: Duarte et al., 2021; Troscianko et al., 2021).

For other variables, to test whether the observed variations are due to temperature treatments, and in comparison with wild individuals, we fitted linear mixed models with the package nlme (Pinheiro et al., 2023), with backward variable selection based on a p-value fixed at 0.05. Weight and sex were added as a variable in the models, where applicable (see results tables). The identity of the wing on which the spectrophotometry measurement was taken was added as a fixed factor in the absorption analyses. In the temperature tolerance tests, the mean temperature and the standard deviation of the temperature in the jars during the trials were added as factors in the statistical analysis. Mother's identity was implemented as a random factor. The parameter for fixed effects in the final models were calculated using Wald tests for the terms in the model giving a F-values of each fixed effect, numerator and denominator degrees of freedom, and the associated p-value significance, with the function anova.lme of the package nlme (Pinheiro et al., 2023). To compare effects between treatments, we used post-hoc pairwise comparison tests with the function emmeans, from the package emmeans in R (Searle et al., 1980), which implements a p-value adjustment with a tukey method for comparing the estimates.

3. Results

Effect of temperature on developmental parameters

The temperature treatment affected the development rate of larvae of both species (*M. confusa*: Table S1a,b, and Figure 3; *T. harmonia*: Table S2a,b, and Figure 3). The higher the temperature, the shorter the time from laying to emergence, with a difference of 19 days on average between 18°C and 27°C treatments for *M. confusa*, and 32 days for *T. harmonia*.

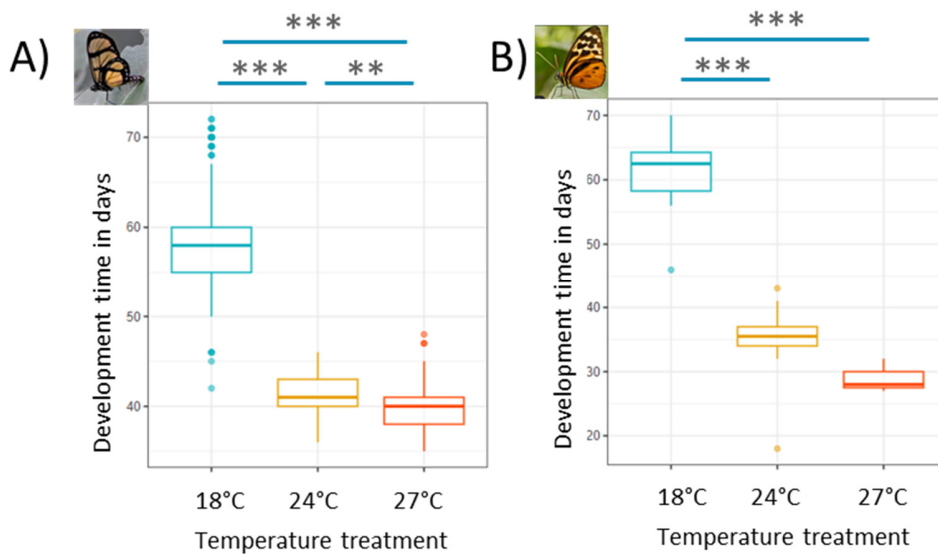


Figure 3. Development time in days of the butterflies, from laying date to emergence, as a function of the temperature treatments. The significance shown here correspond to the associated p-value of the contrast in the pairwise combinations (*P < 0.05; **P < 0.01; ***P < 0.001). A) *M. confusa* (N 18°C = 189, N 24°C = 153, N 27°C = 109). B) *T. harmonia*. (N 18°C = 12, N 24°C = 22, N 27°C = 3).

The coxme model showed that in *M. confusa*, temperature along with laying date affected the survival rate. The most advantageous treatment for survival was the lowest, at 18°C, with 75% of individuals having reached emergence, compared with 58% at 24°C and less than 25% for the 27°C treatment. The L3 and L4 stages were the most sensitive (*M. confusa*: Table S3a,b, and Figure S1).

In *T. harmonia*, the coxme model showed that temperature treatments, as well as calendar date, significantly affected survival. The most advantageous treatment for survival was the one at 24°C with 30% survival expectancy, while the least advantageous treatments were those at 18°C and 27°C with a survival expectancy of 12% and 2% respectively. Early stages, between egg and L3, were the most sensitive. For both species, the best Coxme model took into account the random effect of maternal identity (*T. harmonia*: Table S4a,b, and Figure S1).

The larval weight at the fifth instar was negatively correlated with the temperature, with on average heavier larvae at 18°C than at any other temperature (*M. confusa*: Table S5a,b, and Figure S2 ; *T. harmonia* : Table S6a,b, and Figure S2). It was also the case for the adult dry weight (*M. confusa*: Table S7a,b, and Figure 4 ; *T. harmonia* : Table S8a,b, and Figure 4), the 18°C *M. confusa* were on average 0.159 g heavier than those reared at 27°C, but there was not significant difference with the wild individuals . In *T. harmonia*, the adult weight after the temperature treatments was significantly lower than that of the butterflies in the wild.

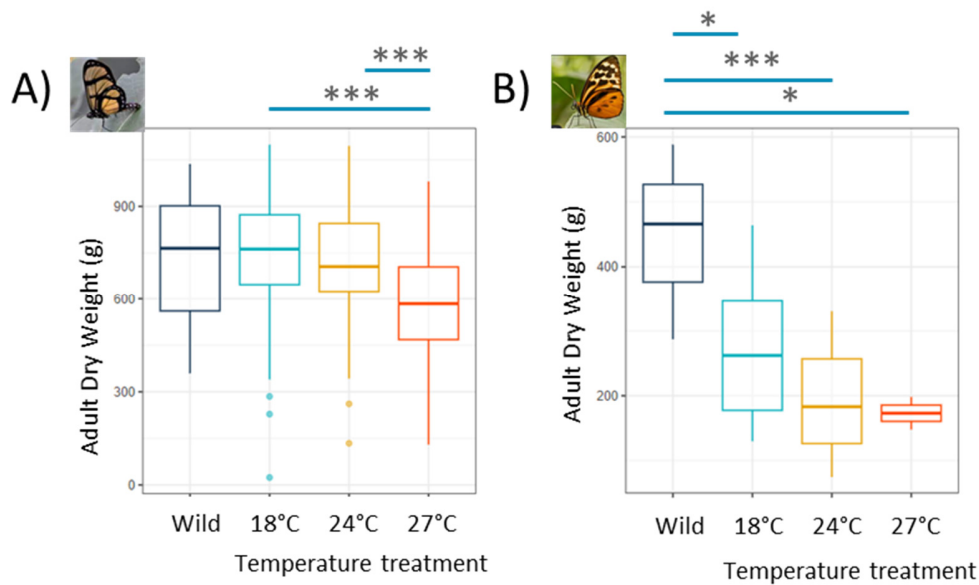


Figure 4. Adults dry weight as a function of the temperature treatments. The significance shown here correspond to the associated p-value of the contrast in the pairwise combinations (*P < 0.05; **P < 0.01; ***P < 0.001). A) *M. confusa* (N Wild = 19, N 18°C = 134, N 24°C = 111, N 27°C = 85). B) *T. harmonia* (N Wild = 3, N 18°C = 11, N 24°C = 21, N 27°C = 2).

Effect of temperature on the wing traits

We found no significant effect of temperature treatment on the wing absorption capacities and degree of darkness in any of the species, nor did we find differences with the wild population. The proportion of dark surface on the wings of the imagoes, whether in *M. confusa* or *T. harmonia*, was not significantly predicted by temperature treatment (*M. confusa*: Table S9, and Figure S3; *T. harmonia* : Table S10, and Figure S3).

Likewise, there was no effect of rearing temperature on total UV-Visible-NIR (300-2500nm) absorption (*M. confusa*: Table S11, and Figure S4-S5 ; *T. harmonia* : Table S12, and Figure S4-S5), nor on separated UV-Visible (300-700 nm) absorption (*M. confusa*: Table S13, and Figure S6 ; *T. harmonia* : Table S14, and Figure S6) and NIR (700-2500 nm) absorption (*M. confusa*: Table S15, and Figure S7; *T. harmonia* : Table S16, and Figure S7). The identity of the wing on which the measurement was taken, hindwing or forewing, was the only factor

predicting the optical properties. Similarly, the *M. confusa* wing transparency, measured as the transmission of the wing spots in the UV-Visible (300-700 nm), was not significantly affected by rearing temperature (*M. confusa*: Table S17 and Figure S8).

Effect of temperature treatment on thermal tolerance

The thermal tolerance tests showed a part of thermally induced effect on the response of the butterflies to temperature shock assays. The cold shock experiment showed a significantly negative association between rearing temperature and the time until knock-out in *M. confusa*: the higher the temperature treatment the quicker they fell into knock-out state, with an average difference of 55 seconds between the two extreme treatments (*M. confusa*: Table S18a,b, and Figure 5). The reared butterflies showed a better resistance to cold knock out than wild-caught butterflies, except for 27°C for which the difference was not significant. *T. harmonia* did not show any significant association between temperature and cold knock-out, despite a trend in the same direction as *M. confusa*. However, wild individual were significantly more resistant than individuals reared at 24°C (*T. harmonia*: Table S19a,b, and Figure 5). Note that statistical power for comparisons involving the 27°C treatment is considerably reduced in *T. harmonia*, because only 2 individuals survived that treatment.

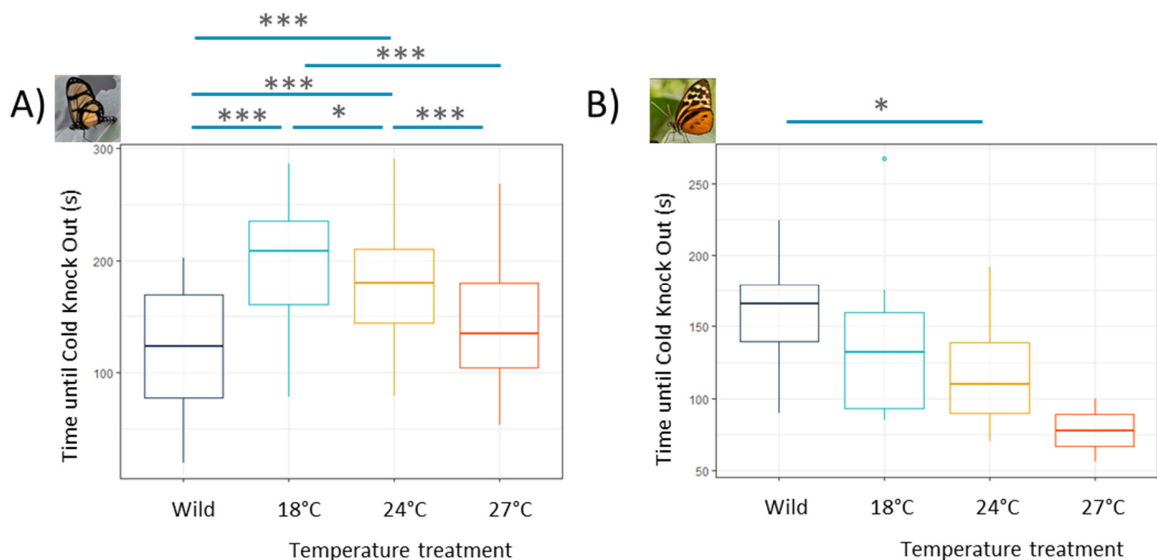


Figure 5. Time until knock-out under cold stress (5°C) in function of the temperature treatments. The significance shown here correspond to the associated p-value of the contrast in the pairwise combinations (*P < 0.05; **P < 0.01; ***P < 0.001). A) *M. confusa* (N Wild = 24, N 18°C = 135, N 24°C = 111, N 27°C = 86) B) *T. harmonia* (N Wild = 8, N 18°C = 11, N 24°C = 21, N 27°C = 2).

The cold shock recovery time was significantly longer for the *M. confusa* reared at 27°C than for the other treatments, but did not differ from wild butterflies (*M. confusa*: Table S20a,b and

Figure S9). We did not detect any significant effect in *T. harmonia*, although the individuals reared at 27°C also took longer to recover (*T. harmonia*: Table S21 and Figure S9).

Contrary to the cold shock experiment, we did not detect any effect of treatment nor any difference with the wild butterflies in the heat knock-out assays. The time until knock out was not significantly different between the groups (*M. confusa*: Table S22 and Figure 6; *T. harmonia*: Table S23 and Figure 6).

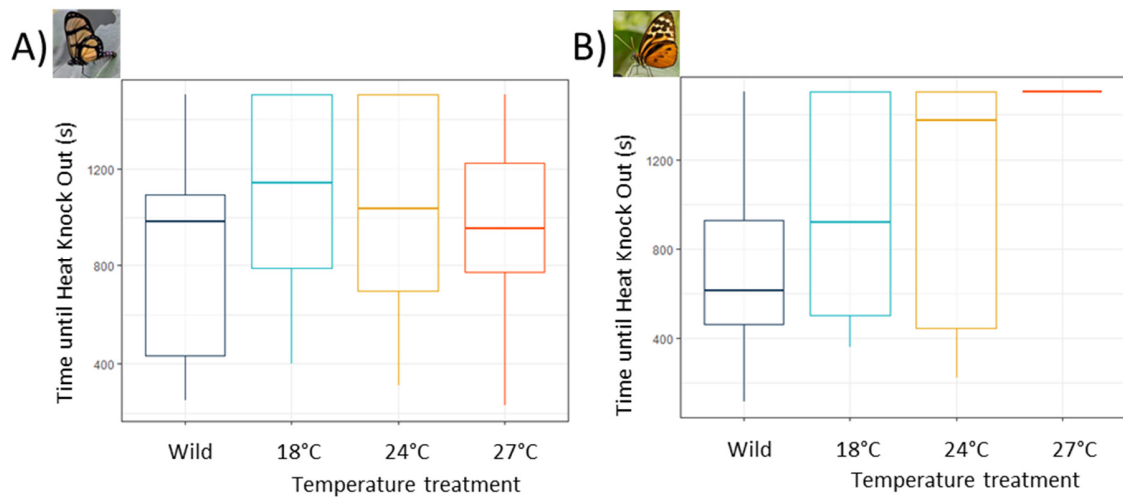


Figure 6. Time until knock-out under heat stress (39°C) in function of the temperature treatments. A) *M. confusa* (N Wild = 23, N 18°C = 135, N 24°C = 108, N 27°C = 84). B) *T. harmonia* (N Wild = 8, N 18°C = 11, N 24°C = 20, N 27°C = 2).

4. Discussion

This study aimed to evaluate the impact of ambient temperature during development on development time, growth and survival, and on traits related to thermal adaptation. This allowed us to assess the extent of plasticity in trait variation in response to different temperature. We found strong effect of temperature on development time, growth and survival during immature stages, but variable effects in the response of physiological and metabolic traits relating to thermoregulation in adults to temperature, with notable differences between the two species studied.

Higher temperatures induce faster development, smaller weight, and increased mortality in immatures

We found a negative effect of high temperatures on the survival of immature stages, confirming previous studies on other tropical (Huang et al., 2022) or temperate (Nylín et al., 1989; Fischer and Karl, 2010) butterfly species. The effect is even more marked in *T. harmonia*, where only two individuals reached the imago stage under the 27°C treatment. Generally, mortality rate was very important at all stages in this species. High temperatures cause irreversible damages to exposed individuals, such as protein denaturation, or impact on neurophysiological functions (King and MacRae, 2015; O’Sullivan et al., 2017). Moreover, thermal stress experienced by larvae likely has carry-over effects on fitness in the longer term, not explored here, which affects adults (Klockmann et al., 2017). Such long-term effects include a drop in fecundity and immune deficiencies, documented in invertebrates living in various environments (Fischer et al., 2014; Jourdan et al., 2019; Awde et al., 2023). In *Pieris napi* (Pieridae), pre-winter thermal conditions affect the fitness of the individual even after the diapause, months later (Nielsen et al., 2022). In addition, immature stages can survive across a relatively large array of temperatures, yet some of those temperatures might fall outside the range of optimal temperature for reproduction and development (Zhang et al., 2015).

We observed other direct consequences on temperature on developmental parameters. Development time was notably shorter in high treatment and both larvae and imagoes had lower body weight with higher temperature treatment. In ectotherms, larger individuals often have better fitness, especially in females, for which there is a positive association between body weight and fecundity (Blanckenhorn, 2000). In fact, the temperature-size rule, which ‘works’ as a thermal reaction norm rule for this physical traits in ectotherms, predicts that the size follows a negative slope with the development temperature, meaning that hotter developmental conditions lead to smaller individuals (Angilletta and Dunham, 2003; Gibert et al., 2004; Kingsolver and Huey, 2008). However, this does not mean that cold temperatures are the most favorable, as one of the costs in having a large body lies in a longer development time, leading inevitably to a later sexual maturation, and the general trend is thus more toward an intermediate size stabilization (Atkinson, 1994, 199; Kingsolver and Huey, 2008).

In *T. harmonia*, the fact that we found an important difference between the weight of wild butterflies and of butterflies reared in the lab, regardless the treatment, might indicate an underlying metabolic stress experienced by the individuals during the rearing in controlled conditions. Such a pervasive difference was not observed in *M. confusa*. Although larval food intake has not been quantified here, it is likely that cold conditions are more favorable to food accumulation and mass gain (Fischer and Karl, 2010). However, in natural conditions climate variability can cancel out the effects of heat stress experienced at a given time, as reported in *Pieris napi* (Greiser et al., 2022).

No plasticity for wing melanization and optical properties in response to temperature experienced during immature development

In emerging butterflies, we examined the phenotypic plasticity of wing morphological traits linked to thermoregulation. The results of spectrophotometric absorption measurements showed that there was no influence of development temperature on wing absorption in the following ranges: UV-Visible, NIR and the total UV-Visible-NIR absorption, even when compared to wild populations. This is a surprising result, especially for the absorption of NIR, which are crucial for heat absorption capacities, since they are not involved in any other aspect of their ecology, being invisible to butterflies and their predators. Similar to the absorption results, the study of the melanic surface area on the wings show that this traits was not influenced by temperature treatments during development either. While no data is available on any Ithomiini and clearwing butterflies, we know that these results depart from what is expected in such ectotherms. Most of the studies testing color variation effect of temperature found the wings to be darker in cooler conditions, with a clear plastic response to temperature (Kingsolver and Huey, 1998; Markl et al., 2022).

Similarly, wing transparency in *M. confusa*, measured as the transmission in the UV-Visible range (the wavelength range that is relevant for visual interactions with other species), provide the first insight of the influence of developmental conditions on wing features in clearwing butterflies, and our results suggest that there is no such association. Further work needs to be carried out on multiple species spanning a large range in wing colorations, from fully opaque to highly transparent, to test whether transparency extent can vary with rearing temperature in different transparency contexts. All these results indicate the low adaptative potential, as far as thermal requirements is concerned, of wing physiology in ithomiine butterflies. This is in line with what was found in a previous study on Ithomiini transparency (Ossola et al., 2023), where we found no link between wing coloration, thermal properties or absorption capacities of the UV-Visible and NIR ranges and the climatic conditions in their natural habitat. A possible explanation for the absence of apparent response of wing coloration or thermal properties to temperature variations is that coloration in ithomiine butterflies is most likely primarily under selection from predation pressure, with clearwing species, albeit

aposematic (Pinna et al., 2021) being more cryptic to predators (Arias et al., 2019; McClure et al., 2019).

Adaptive plastic thermal tolerance to cold temperatures

When we explored the plasticity of the thermal tolerance of adult butterflies to extreme cold-stress, we found that cold resistance is significantly influenced by the temperature experienced during immature stages. Individuals reared at cooler temperatures have greater tolerance to cold, i.e. a later knockout and a faster ability to recover from cold shock. While this effect is statistically significant for *M. confusa*, it is not the case for *T. harmonia*. This is most likely because the latter were less resistant to the rearing conditions in general, and most specifically for *T. harmonia* reared at 27°C, which have turned out to be particularly fragile, greatly influencing their poor score in both thermal tolerance tests.

In *M. confusa*, the negative correlation between developmental temperature and cold knock out time indicates underlying adaptive plasticity. Plus, wild butterflies are less tolerant than butterflies reared at 18°C, proving that, for cold, performance can be improved if butterflies are exposed to cold conditions during the larval phase. Our heat test results show no significant link between developmental temperature and heat-stress tolerance in adults, and we can conclude that we did not detect adaptive plastic effect of developmental treatment. Our results depart from observations of other Lepidoptera, where prolonged, or sometimes even acute, exposure to high temperatures can result in adaptive plasticity of upper thermal limits and heat tolerance (Karl et al., 2008; Fischer and Karl, 2010; Kingsolver et al., 2016). However, a review of the temperature acclimation in invertebrate and vertebrate ectotherms showed that in general, upper thermal limit have a limited acclimation response (Gunderson and Stillman, 2015).

In a previous study of Ecuadorian ithomiine species, we found that species native to high altitudes were more resistant to cold stress, but less resistant to heat stress, than low-altitude species (chapter 2). This effect persisted after accounting for phylogenetic relationships, suggesting that the thermal response stemmed from either genetic adaptation, either from plasticity. Here we showed that the origin of the adaptive response to cold conditions we observed in Panama and in the Andes probably lies in the association between genetics and plasticity. These promising results should be developed further by making common garden rearing of ithomiine butterflies from different altitude to compare the response of genetically distinct species and populations (Montejo-Kovacevich et al., 2020).

Ectotherm communities in the face of climate change

Our study highlighted deleterious effects of high temperature, especially in immature stages, on fitness-related traits. Climate change, through increasing temperatures and more extreme climatic events, is expected to shift the distribution range of many plant and animal taxa

upward and toward the poles (Parmesan et al., 1999; Kelly and Goulden, 2008; McCain and Garfinkel, 2021). Environmentally-induced plasticity can be as important as genetic factors in adaptive responses of ectotherms to temperature stress, providing a potentially important offset to the negative consequences of rising temperatures. While our study shows that cold tolerance is potentially plastic, heat tolerance was not affected by the developmental temperature. This may be linked to the observation that in most vertebrate and invertebrate species, the plastic effect is less pronounced for upper limits than on lower ones (Hoffmann et al., 2013). Indeed, in many arthropod taxa the potential of adaptive plasticity, which entails a reduction of the effects of heat stress and an increase in heat tolerance significantly, may turn out to be limited (Hoffmann et al., 2013; Gunderson and Stillman, 2015). For instance, the increases in critical maximum temperature of some *Drosophila* species are restricted only up to 0.6–1.0°C (Kellermann and Sgrò, 2018) and 1–2°C for the moth *Manduca sexta* (Kingsolver et al., 2016). For most ectothermic taxa, vertebrates and invertebrates, upper thermal tolerance barely increases or is non-existent as the thermal challenge increases (Gunderson and Stillman, 2015). Plus, response to heat stress can be associated with other effects that are indirectly related but have a cost on fitness, such as declines in offspring size and survival, or immunity (Kingsolver and Huey, 2008; Fischer et al., 2014).

Variations in thermal trait exist at every stage of the life cycle, and documenting them is necessary to determine populations and species potential to cope with a changing environment. Specifically, mechanisms involved in temperature buffering in immature stages remain to be explored. Thermal behavior of the larvae, such as the preferred microclimate refuges offered by the vegetation can be crucial to withstand periods of heat stress (Kearney et al., 2009; Woods et al., 2015; Pincebourde and Suppo, 2016). Adaptive plasticity has variable effects depending on the habitat, latitude, altitude and thermal seasonality in which the organisms live (Gunderson and Stillman, 2015). High latitude species of *Drosophila* sp. from cooler climates are characterized by larger plasticity of thermal performances, as such species have adapted to greater variability in environmental temperatures (Maclean et al., 2019). In butterflies in the Lycaenidae family, low-altitude populations have a higher plasticity in heat stress tolerance than high altitude ones, which makes the latter less able to withstand temperature rising (Fischer and Karl, 2010).

While the response to rising temperatures is important to consider, it is also clear that since species will increasingly shift their range upwards or towards high latitudes, i.e. colder, more favorable climates, they expose themselves to the risk of occasional cold stress (Halsch et al., 2021). Low thermal limits are often related to the geographical distribution of organisms, as they decrease with increasing altitude and latitude (Sinclair et al., 2012). In *Pieris manni*, comparison of implanted and newly shifted populations showed that newly introduced populations have a higher plasticity for cold tolerance that enable them to cope with unfamiliar conditions (Neu and Fischer, 2022).

Conclusion

Developmental conditions are critical to the physiology and survival of immature stages, and the high temperatures had a great risk of survival. Temperature had a contrasting effect on wing morphology and thermal tolerance in adult butterflies. The temperature treatment had no effect on the optical properties and degree of darkness of the wings, while resistance to cold was greater if they were reared at cooler temperatures. On the other hand, development had no effect on heat tolerance. Thermal adaptation in butterflies probably results from a combination of genetic and plastic effects, and it is difficult to estimate what will be their implications in resilience to climate change.

Aknowledgments

This work was funded by Clearwing ANR project (ANR-16-CE02-0012), HFSP project on transparency (RGP0014/2016), AAP departement Origine et Evolution of MNHN (TOLEATHERM). VO is recipient of Short Term Fellowship from the Smithsonian Tropical Research Institute, and a doctoral fellowship from the doctoral school FIRE, Learning Planet Institute. Authorities of Panama granted collection permit SE/A-82-19 to OM ; and export permit PA-01-ARB-076-2022 to VO. We thank the STRI staff in Gamboa and Tupper for the lab access, equipment, authorizations and permits. We warmly thank Esme Ashe-Jepson and Greg Lamarre for letting us access their laboratory and use the equipment, Oscar Paneso and Cruz Batista for their work in the STRI insectarium, Joe Hanly for the Insightful discussion on butterflies and the help with the export permits, Jean-Pierre Vesco for sending Greta oto eggs and his expertise on butterfly rearing, and the fellow students of the butterfly team involved in the insectarium maintenance.

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Chapter 3 – Appendix

Supplementary Figures and Tables

Table S1a. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the developmental duration, from laying date to emergence, in days, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
Development time (days) <i>M. confusa</i>	(Intercept)	15703.652 (1, 439)	<.001***
	Treatment group	1151.001 (2, 439)	<.001***

Table S1b. Results of the pairwise contrasts combinations between treatments estimated from the final mixed-effect model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Treatment group contrasts	estimate	standard error	df	t.ratio	p-value
Development time (days) <i>M. confusa</i>	18°C - 24°C	17.47	0.433	439	40.357	<.001***
	18°C - 27°C	19.04	0.478	439	39.826	<.001***
	24°C - 27°C	1.57	0.498	439	3.148	<.001***

Table S2a. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the developmental duration, from laying date to emergence, in days, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
Development time (days) <i>T. harmonia</i>	(Intercept)	564.6673 (1, 31)	<.001***
	Treatment group	135.214 (2, 31)	<.001***

Table S2b. Results of the pairwise contrasts combinations between treatments estimated from the final mixed-effect model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Treatment group contrasts	estimate	standard error	df	t.ratio	p-value
Development time (days) <i>T. harmonia</i>	18°C - 24°C	26.59	1.8	31	14.78	<.001***
	18°C - 27°C	32.450	3.130	31	10.371	<.001***
	24°C - 27°C	5.850	3.000	31	1.948	0.143

Table S3a. Results of the retained coxme model after backward elimination of the fixed parameters, testing the effect of treatments on the survival rate, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Fixed parameter	regression beta	coefficients	standard error	z.ratio	p-value
Treatment 24°C	-1.642		0.729	-2.252	0.024*
Treatment 27°C	2.697		0.564	4.778	<.001***
Laying date	-0.013		0.012	-1.008	0.313
Treatment 24°C x Laying date	0.052		0.016	3.175	<.001**
Treatment 27°C x Laying date	-0.023		0.013	-1.730	0.083

Table S3b. Results of the pairwise contrasts combinations between treatments estimated from the final coxme model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Treatment group contrasts	estimate	standard error	z.ratio	p-value
18°C - 24°C	-0.59	0.161	-3.657	<.001***
18°C - 27°C	-1.71	0.137	-12.497	<.001***
24°C - 27°C	-1.12	0.115	-9.753	<.001***

Table S4a. Results of the retained coxme model after backward elimination of the fixed parameters, testing the effect of treatments on the survival rate, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Fixed parameter	regression beta	coefficients	standard error	z.ratio	p-value
Treatment 24°C	-0.9789		0.1777	-5.5099	<.001***
Treatment 27°C	-0.1321		0.1440	-0.9177	0.358
Laying date	0.0630		0.0163	3.8714	<.001***
Other factors not retained : Treatment 27°C x Laying date					

Table S4b. Results of the pairwise contrasts combinations between treatments estimated from the final coxme model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Treatment group contrasts	estimate	standard error	z.ratio	p-value
18°C - 24°C	0.979	0.178	5.51	<.001***
18°C - 27°C	0.132	0.144	0.918	0.3588
24°C - 27°C	-0.847	0.166	-5.109	<.001***

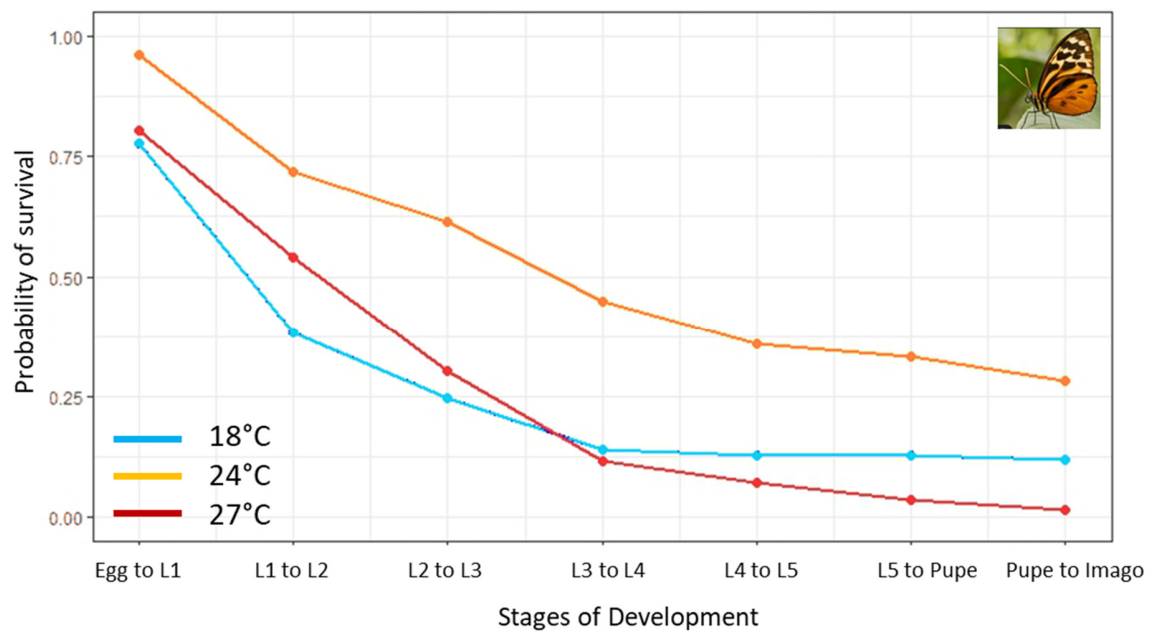
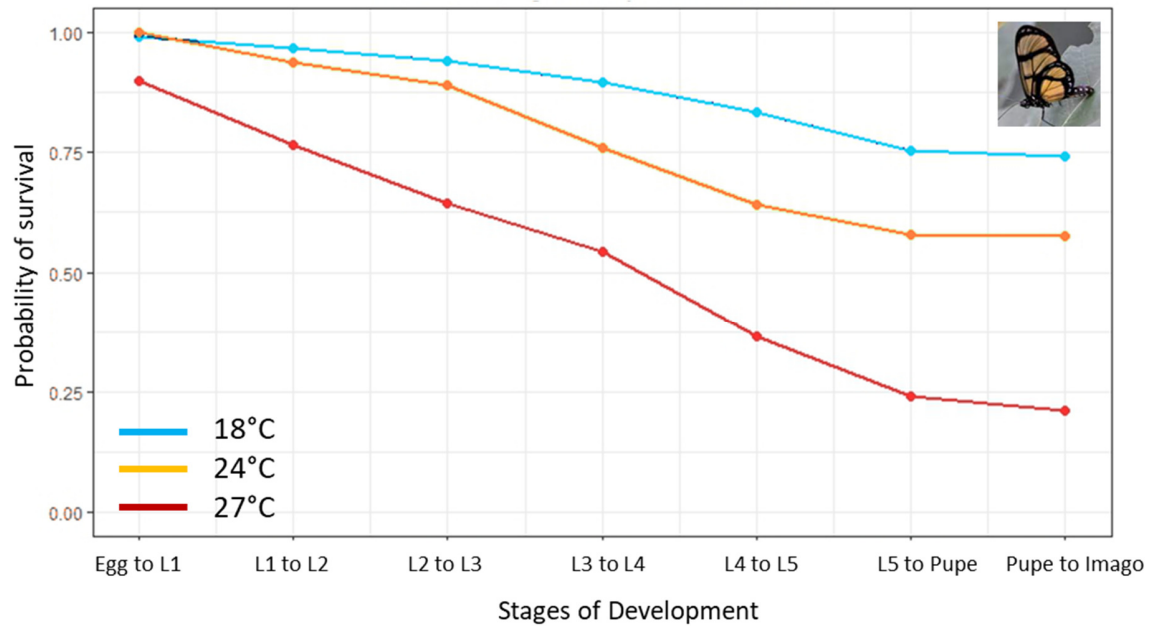


Figure S1. Probability of survival in function of developmental stage in *M. confusa* (top) and *T. harmonia* (bottom), from laying date to emergence, in function of the temperature treatments.

Table S5a. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the larval weight at fifth instar, in grams, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
L5 weight (g) <i>M. confusa</i>	(Intercept)	2531.3126 (1, 606)	<.001***
	Treatment group	13.5332 (2, 606)	<.001***

Table S5b. Results of the pairwise contrasts combinations between treatments estimated from the final mixed-effect model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Treatment group contrasts	estimate	standard error	df	t.ratio	p-value
L5 weight (g) <i>M. confusa</i>	18°C - 24°C	0.036	0.014	606	2.516	0.033*
	18°C - 27°C	0.071	0.014	606	5.189	<.001***
	24°C - 27°C	0.035	0.014	606	2.521	0.032*

Table S6a. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the larval weight at fifth instar, in grams, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
L5 weight (g) <i>T. harmonia</i>	(Intercept)	185.39188 (1, 36)	<.001***
	Treatment group	5.69576 (2, 36)	<0.05*

Table S6b. Results of the pairwise contrasts combinations between treatments estimated from the final mixed-effect model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Treatment group contrasts	estimate	standard error	df	t.ratio	p-value
L5 weight (g) <i>T. harmonia</i>	18°C - 24°C	0.0809	0.05	36	1.6190	0.251
	18°C - 27°C	0.2044	0.063	36	3.244	<0.05*
	24°C - 27°C	0.1235	0.0542	36	2.276	0.072

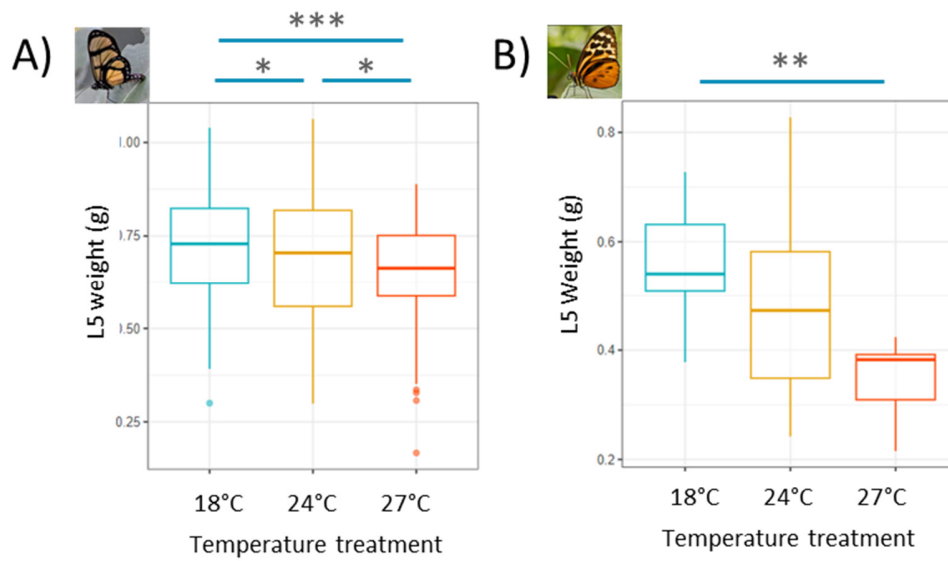


Figure S2. Larval weight at fifth instar as a function of the temperature treatments. The significance shown here correspond to the associated p-value of the contrast in the pairwise combinations (*P < 0.05; **P < 0.01; ***P < 0.001). A) *M. confusa* (N 18°C = 205, N 24°C = 186, N 27°C = 227) B) *T. harmonia* (N 18°C = 12, N 24°C = 24, N 27°C = 8).

Table S7a. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the adults dry weight, in grams, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
dry weight (g) <i>M. confusa</i>	(Intercept)	30.121871 (1, 317)	<.001***
	Treatment group	12.16666 (3, 317)	<.001***
	Sex	5.889865 (1, 317)	0.015*
	Treatment x Sex	5.171565 (3, 317)	<.001***

Table S7b. Results of the pairwise contrasts combinations between treatments estimated from the final mixed-effect model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Treatment group contrasts	estimate	standard error	df	t.ratio	p-value
dry weight (g) <i>M. confusa</i>	WILD - 18°C	-101.1	54.1	317	-1.871	0.242
	WILD - 24°C	-74.4	54.5	317	-1.367	0.521
	WILD - 27°C	58	55.2	317	1.052	0.719
	18°C - 24°C	26.7	20.9	317	1.277	0.578
	18°C - 27°C	159.2	22.8	317	6.981	<.001***
	24°C - 27°C	132.5	23.9	317	5.543	<.001***

Table S8a. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the adults dry weight, in grams, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
dry weight (g) <i>T. harmonia</i>	(Intercept)	30.121871 (1, 26)	<.001***
	Treatment group	133.253 (3, 26)	<.001***
	Sex	9.72178 (1, 26)	<.001***
Other factors not retained : Temperature treatment x Sex			

Table S8b. Results of the pairwise contrasts combinations between treatments estimated from the final mixed-effect model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Treatment group contrasts	estimate	standard error	df	t.ratio	p-value
dry weight (g) <i>T. harmonia</i>	WILD - 18°C	120.1	43.9	26	2.737	0.050*
	WILD - 24°C	191.6	39.6	26	4.839	<.001***
	WILD - 27°C	223.9	76.1	26	2.94	0.032*
	18°C - 24°C	71.5	35.5	26	2.017	0.207
	18°C - 27°C	103.8	74.4	26	1.395	0.514
	24°C - 27°C	32.3	72.3	26	0.447	0.970

Table S9. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the proportion of Dark coloration on the wings, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
Proportion of dark coloration <i>M. confusa</i>	(Intercept)	4909.207 (1, 319)	<.001***
Other factors not retained : Temperature treatment, Sex, Temperature treatment x Sex			

Table S10. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the proportion of Dark coloration on the wings, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
Proportion of dark coloration <i>T. harmonia</i>	(Intercept)	532.2288 (1, 29)	<.001***
Other factors not retained : Temperature treatment, Sex, Temperature treatment x Sex			

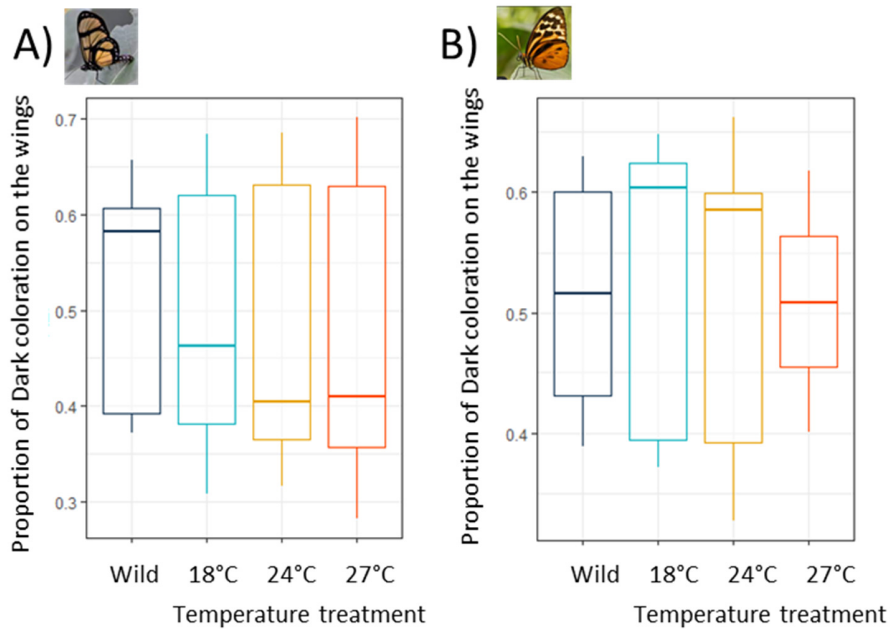


Figure S3. Proportion of dark coloration on the wings as a function of the temperature treatments. A) *M. confusa* (N Wild = 20, N 18°C = 131, N 24°C = 114, N 27°C = 80) B) *T. harmonia* (N Wild = 8, N 18°C = 11, N 24°C = 22, N 27°C = 2).

Table S11. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the wing spots UV-Visible-NIR absorption, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
UV-Visible-NIR absorption <i>M. confusa</i>	(Intercept)	2797.248 (1 , 164)	<.001***
	Wing	128.832 (1 , 164)	<.001***
Other factors not retained : Temperature treatment, Sex, Temperature treatment x Sex			

Table S12. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the wing spots UV-Visible-NIR absorption, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
UV-Visible-NIR absorption <i>T. harmonia</i>	(Intercept)	1985.878 (1 , 26)	<.001***
	Wing	293.4008 (1 , 26)	<.001***
Other factors not retained : Temperature treatment, Sex, Temperature treatment x Sex			

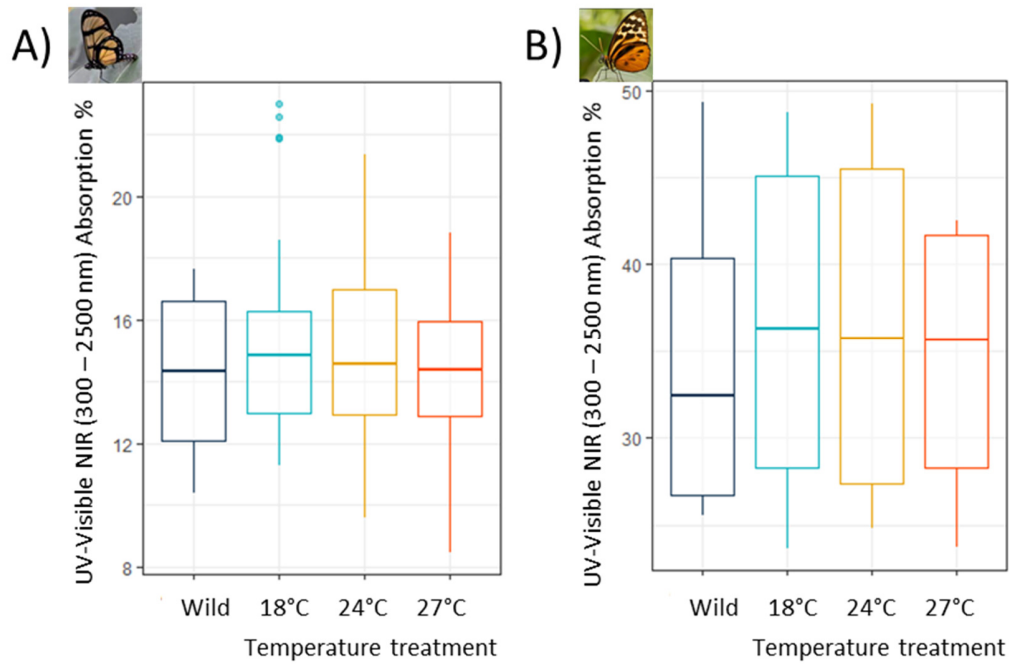


Figure S4. Wing spots UV-Visible-NIR (300-2500 nm) absorption in function of the temperature treatments. A) *M. confusa* (N Wild = 16, N 18°C = 56, N 24°C = 56, N 27°C = 52) B) *T. harmonia* (N Wild = 8, N 18°C = 10, N 24°C = 12, N 27°C = 4).

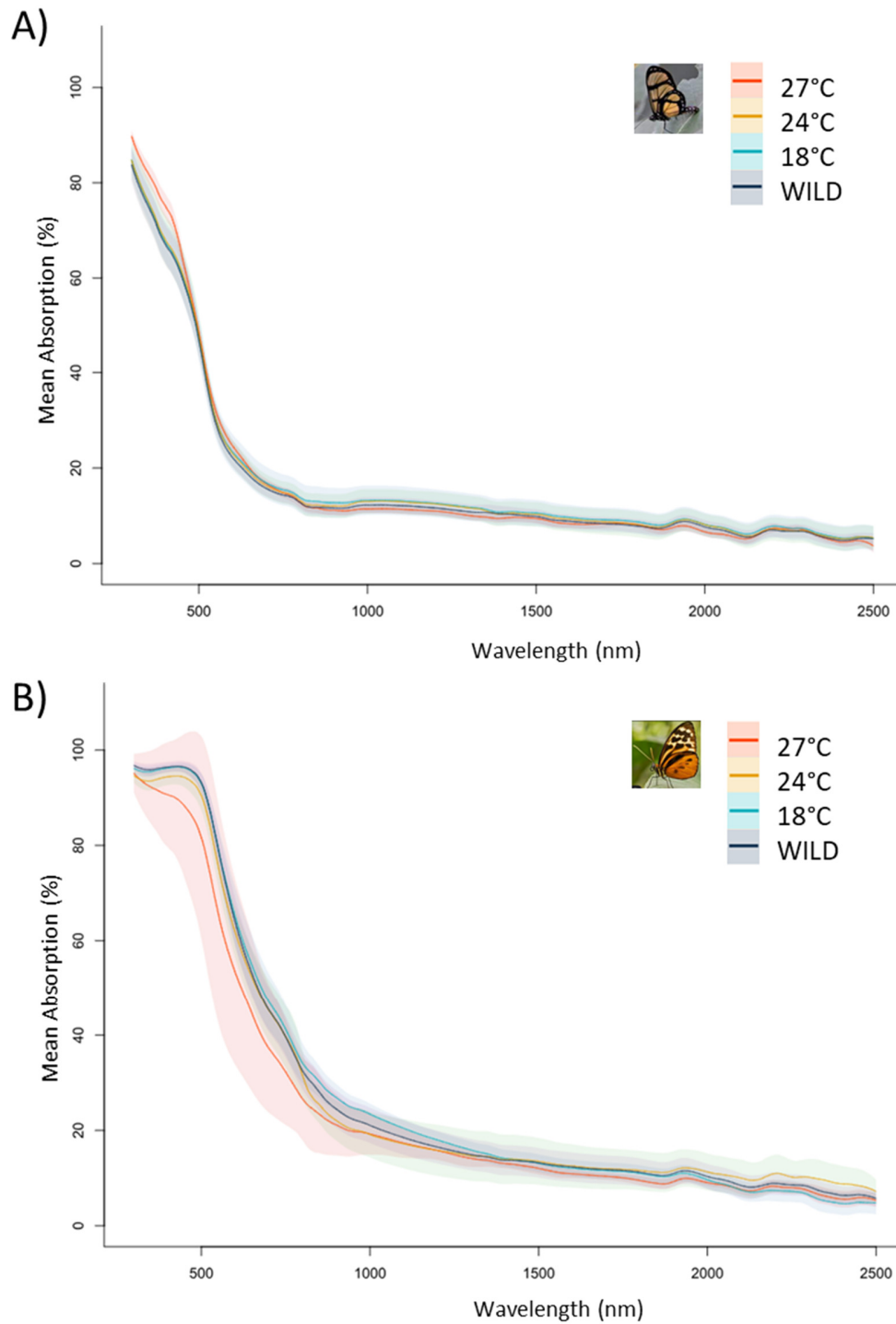


Figure S5. Mean absorption spectra and 95% standard deviation of wing spots UV-Visible-NIR (300-2500 nm) absorption, for A) *M. confusa* B) *T. harmonia*.

Table S13. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the wing spots UV-Visible absorption, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
UV-Visible absorption <i>M. confusa</i>	(Intercept)	6883.174 (1 , 164)	<.001***
	Wing	326.55 (1 , 164)	<.001***
Other factors not retained : Temperature treatment, Sex, Temperature treatment x Sex			

Table S14. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the wing spots UV-Visible absorption, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
UV-Visible absorption <i>T. harmonia</i>	(Intercept)	24199.083 (1 , 26)	<.001***
	Wing	285.147 (1 , 26)	<.001***
Other factors not retained : Temperature treatment, Sex, Temperature treatment x Sex			

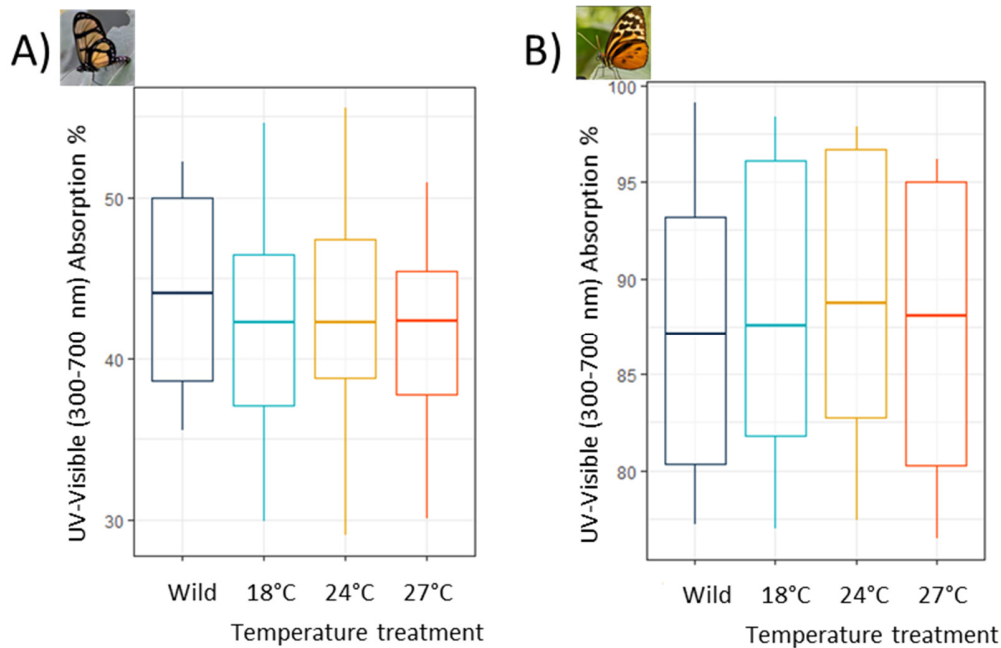


Figure S6. Wing spots UV-Visible (300-700 nm) absorption as a function of the temperature treatments. A) *M. confusa* (N Wild = 16, N 18°C = 56, N 24°C = 56, N 27°C = 52) B) *T. harmonia* (N Wild = 8, N 18°C = 10, N 24°C = 12, N 27°C = 4).

Table S15. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the wing spots NIR absorption, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
NIR absorption	(Intercept)	951.810 (1, 164)	<.001***
<i>M. confusa</i>	Wing	45.961 (1, 164)	<.001***
Other factors not retained : Temperature treatment, Sex, Temperature treatment x Sex			

Table S16. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the wing spots NIR absorption, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
NIR absorption	(Intercept)	1985.878 (1, 26)	<.001***
<i>T. harmonia</i>	Wing	293.4008 (1, 26)	<.001***
Other factors not retained : Temperature treatment, Sex, Temperature treatment x Sex			

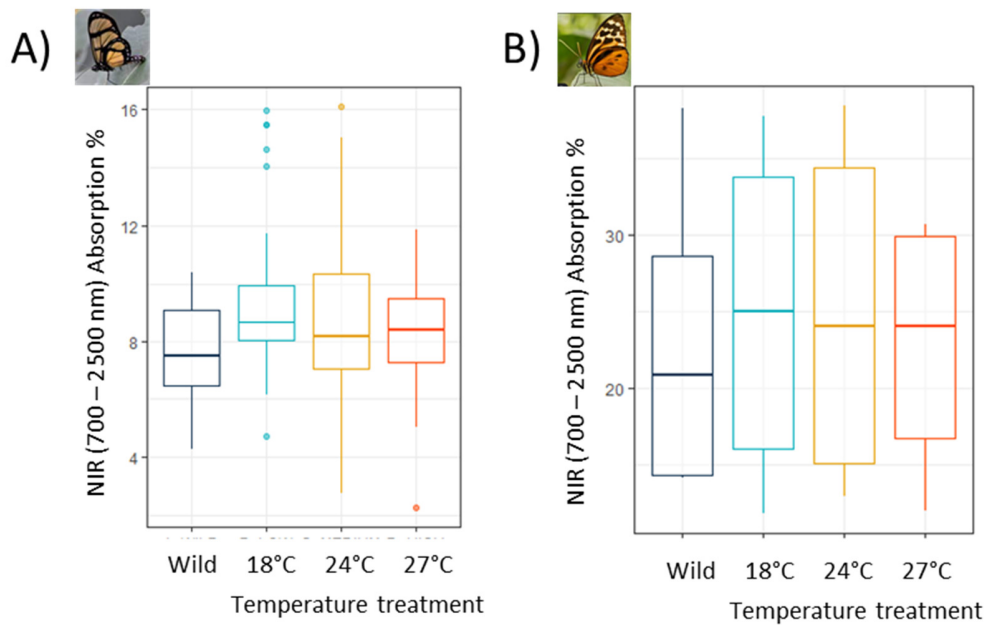


Figure S7. Wing spots NIR (700-2500 nm) absorption as a function of the temperature treatments. The significance shown here correspond to the associated p-value of the contrast in the pairwise combinations (*P < 0.05; **P < 0.01; ***P < 0.001). A) *M. confusa* (N Wild = 16, N 18°C = 56, N 24°C = 56, N 27°C = 52) B) *T. harmonia* (N Wild = 8, N 18°C = 10, N 24°C = 12, N 27°C = 4).

Table S17. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the wing spots UV-Visible transmission, as a proxy for transparency, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
UV-Visible transmission <i>M. confusa</i>	(Intercept)	17039.297 (1, 164)	<.001***
	Wing	333.368 (1, 164)	<.001***
Other factors not retained : Temperature treatment, Sex, Temperature treatment x Sex			

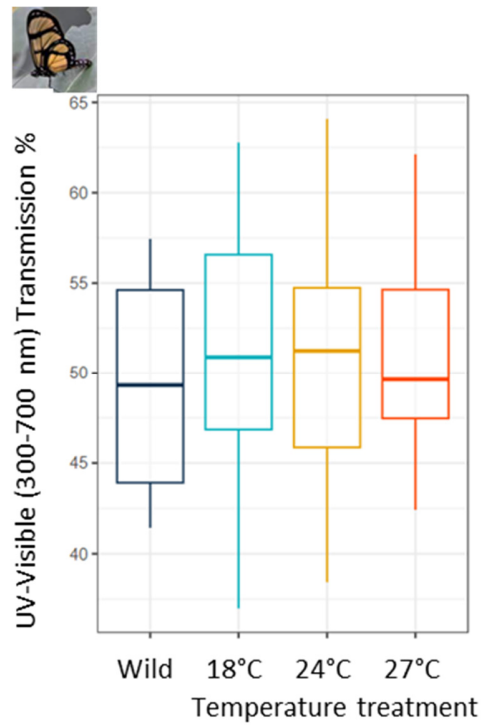


Figure S8. Wing spots UV-Visible (300-700 nm) transmission as a function of the temperature treatments, in *M. confusa* (N Wild = 16, N 18°C = 56, N 24°C = 56, N 27°C = 52).

Table S18a. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the time until knock-out under cold stress (5°C), in seconds, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
Time to COLD knock-out (s) <i>M. confusa</i>	(Intercept)	153.02909 (1, 323)	<.001***
	Treatment group	31.14117 (3, 323)	<.001***
Other factors not retained : Sex, Temperature treatment x Sex, Mean temperature in the jar, Standard deviation of the temperature in the jar			

Table S18b. Results of the pairwise contrasts combinations between treatments estimated from the final mixed-effect model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Treatment group contrasts	estimate	standard error	df	t.ratio	p-value
Time to COLD knock-out (s) <i>M. confusa</i>	WILD - 18°C	-75.1	11.05	323	-6.798	<.001***
	WILD - 24°C	-55.8	11.21	323	-4.983	<.001***
	WILD - 27°C	-20.1	11.5	323	-1.744	0.303
	18°C - 24°C	19.3	6.32	323	3.050	0.013 *
	18°C - 27°C	55.1	6.8	323	8.103	<.001***
	24°C - 27°C	35.8	7.09	323	5.051	<.001***

Table S19a. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the time until knock-out under cold stress (5°C), in seconds, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
Time to COLD knock-out (s) <i>T. harmonia</i>	(Intercept)	141.32422 (1, 28)	<.001***
	Treatment group	4.16851 (3, 28)	0.015*
Other factors not retained : Sex, Temperature treatment x Sex, Mean temperature in the jar, Standard deviation of the temperature in the jar			

Table S19b. Results of the pairwise contrasts combinations between treatments estimated from the final mixed-effect model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Treatment group contrasts	estimate	standard error	df	t.ratio	p-value
Time to COLD knock-out (s) <i>T. harmonia</i>	WILD - 18°C	22.9	18.7	28	1.223	0.618
	WILD - 24°C	45.9	16.7	28	2.747	0.048*
	WILD - 27°C	82.9	31.8	28	2.605	0.066
	18°C - 24°C	23	15	28	1.539	0.428
	18°C - 27°C	60	30.9	28	1.94	0.235
	24°C - 27°C	37	29.8	28	1.241	0.606

Table S20a. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the time until recovery after a cold stress (5°C), in seconds, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
Time to COLD recovery (s) <i>M. confusa</i>	(Intercept)	0.108236 (1, 321)	0.742
	Treatment group	4.597635 (3, 321)	<.001***
	Mean temperature in the jar	5.669069 (1, 321)	0.017
Other factors not retained : Sex, Temperature treatment x Sex, Standard deviation of the temperature in the jar			

Table S20b. Results of the pairwise contrasts combinations between treatments estimated from the final mixed-effect model. The contrasts with a significant associated p-value are indicated in bold. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Treatment contrasts	group estimate	standard error	df	t.ratio	p-value
Time to COLD recovery (s) <i>M. confusa</i>	WILD - 18°C	25.75	21.32	321	1.2080	0.6225
	WILD - 24°C	29.02	21.03	321	1.3800	0.5129
	WILD - 27°C	3.63	21.34	321	0.1700	0.9982
	18°C - 24°C	3.27	7.2	321	0.4550	0.9687
	18°C - 27°C	-22.12	7.47	321	-2.9620	0.017*
	24°C - 27°C	-25.39	7.78	321	-3.2650	<.001**

Table S21. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the time until recovery after a cold stress (5°C), in seconds, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
Time to COLD recovery (s) <i>T. harmonia</i>	(Intercept)	67.39008 (1, 31)	<.001***
Other factors not retained : Temperature treatment, Sex, Temperature treatment x Sex, Standard deviation of the temperature in the jar			

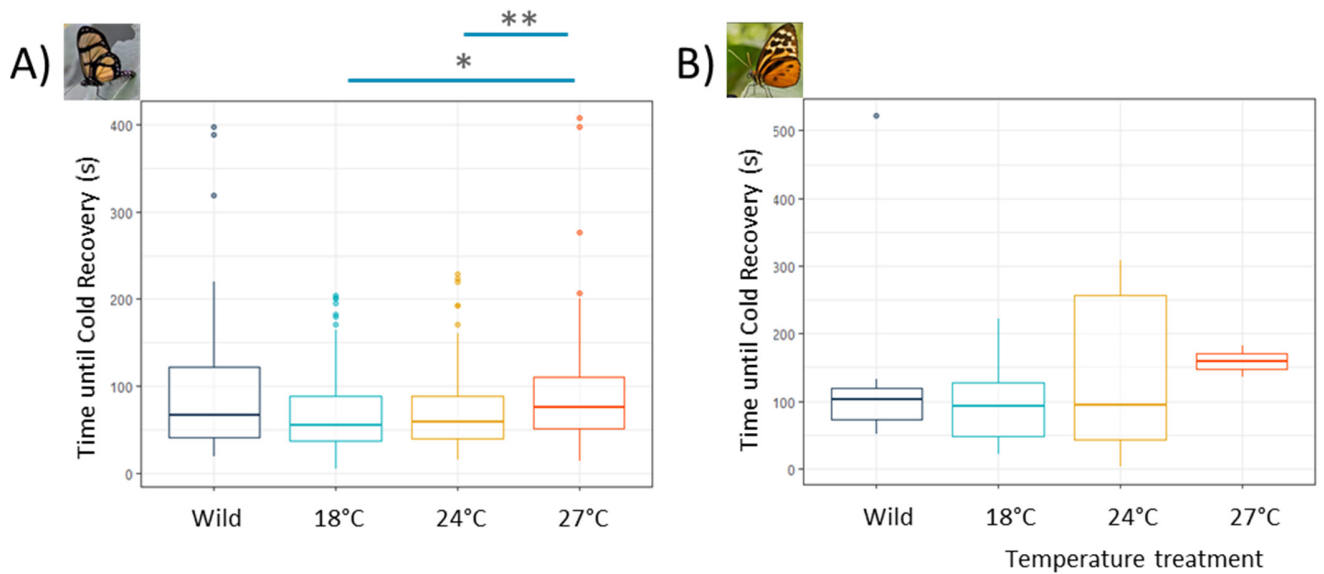


Figure S9. Time until recovery after a cold stress (5°C) in function of the temperature treatments. The significance shown here correspond to the associated p-value of the contrast in the pairwise combinations (*P < 0.05; **P < 0.01; ***P < 0.001). A) *M. confusa* (N Wild = 24, N 18°C = 135, N 24°C = 111, N 27°C = 86) B) *T. harmonia* (N Wild = 8, N 18°C = 11, N 24°C = 21, N 27°C = 2).

Table S22. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the time until knock-out under heat stress (39°C), in seconds, with the associated p-value, for *M. confusa*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
Time to HEAT knock-out (s) <i>M. confusa</i>	(Intercept)	346.0765 (1, 321)	<.001***
	Standard deviation of temperature in the jar	22.9067 (1, 321)	<.001***
Other factors not retained : Temperature treatment , Sex, Temperature treatment x Sex, Mean temperature in the jar			

Table S23. Results of the Wald tests for the terms of the retained linear mixed model after backward elimination of the fixed parameters, testing the effect of treatments on the time until knock-out under heat stress (39°C), in seconds, with the associated p-value, for *T. harmonia*. (*P < 0.05; **P < 0.01; ***P < 0.001).

Dependent variable	Effect	F-value (df num, denum)	p-value
Time to HEAT knock-out (s) <i>T. harmonia</i> .	(Intercept)	22.692032 (1, 29)	<.001***
	Standard deviation of temperature in the jar	8.926677 (1, 29)	<.05*
Other factors not retained : Temperature treatment , Sex, Temperature treatment x Sex, Mean temperature in the jar			

General Discussion

1. Wing properties and thermoregulation in Ithomiini

In our work, we explored how ithomiine butterfly wing thermal properties affect thermoregulation. We assessed whether and how climatic conditions have influenced the evolution of the optical and thermal properties of Ithomiini butterfly wings, and in particular the thermal implications of wing coloration.

Wings are crucial morphological features in butterfly's thermoregulation. Their degree of darkness is directly linked to heat absorption (Watt, 1968): when exposed to the sun, darker butterflies heat up faster, which has direct consequence on the time spent flying (Guppy, 1986). The classic version of the thermal melanism hypothesis, which posits that darker pigmented organisms should be found in colder climates (Clusella Trullas et al., 2007), was not supported by our study. Along the climatic gradient studied in the first chapter, the proportion of melanized surface on the wings becomes lower and lower with increasing elevation, which runs counter to the predictions. This provides further support for observations of the distribution of mimicry rings, whose pattern seemed at first sight to be more transparent at higher altitudes in the Andes (Chazot et al., 2014). Our spectrophotometric and thermal imaging measurements were a real advance in providing information on the wings' actual light-absorbing capacities. Thermal camera measurements revealed that transparent patches contribute very little to light absorption and are poor heat accumulators.

Then, we found no link between optical properties and climatic conditions. For all the species studied, absorption decreases at longer wavelengths. The low absorption in the UV-Visible is not compensated for by the absorption in the NIR, which is low for all species, but remains on average higher for species that are opaque in the human visible range. This was the most surprising result, as NIR absorption, on the contrary to the UV and visible ranges, is mostly subject to thermal adaptation as they cannot be seen by butterflies or their predators (Hart, 2001). We have already mentioned the paucity of existing data on the optical properties of butterfly wings, in this range, and even more in relation to their climatic niche. The recent studies that have set out to measure the link between NIR reflectance and climatic gradients have found that climate is a better predictor of these wavelengths than any other range, with a lower reflectance of NIR in the colder climate (Munro et al., 2019; Kang et al., 2021). However, it should be noted that these studies, like most others on animal coloration, have focused solely on the reflectance properties of insect cuticles. By measuring the absorption

properties of wings from UV to NIR, we were able to show that wings are in general highly transmissive in the NIR, even for opaque species, and that measuring both reflection and transmission is therefore essential. This has to be taken into account in the studies that have focused on measuring the light reflected by the wings, and have most likely measured part of the radiations reflected by the substrate (Munro et al., 2019; Kang et al., 2021). Our results therefore emphasize the need to measure the whole absorption spectra of the cuticles, which results from reflection and transmission spectra, in order to get a more accurate understanding of optical properties.

In chapter 3, we compared the degree of darkness and the optical properties of the wings of butterflies having experienced different developmental conditions (18°C, 24°C, or 27°C), in the opaque *Tithorea harmonia* and the clearwing *Methona confusa*. We found no influence of developmental temperature on wing properties in either of them. This reinforces the conclusion that the relationship between wing colors and thermal adaptation is weak in ithomiine. Color plasticity is probably stronger for temperate species, with a strong seasonal variability. *Junonia coenia* (Nymphalidae), from Northern America, undergoes a strong seasonal plasticity, with dark wings when pupae are put in the cold exposed to a short photoperiod, and paler colors when placed in conditions mimicking the warm season, hot temperatures and long photoperiod (Smith, 1993; Järvi et al., 2019). Environmental signals such as day length or temperature are translated into hormonal signals involved in developmental pathways (Van der Burg and Reed, 2021). The influence of temperature on adult wing color lies in a difference in ecdysone signaling during development, which seems to be the mediator between environmental conditions and wing phenotype in nymphalids, with two periods of ecdysone pulse sensitive in intensity to temperature: in prepupation and during pupation (Oostra et al., 2010). It would be very insightful to try to decouple the key stages of inter-individual thermal responses at different stages of development in tropical butterflies, which are not prone to large seasonal variations.

Imago is not the only stage where coloration may have adaptive plastic potential. As with all holometaboles, immature stages have a totally different life mode than adults, and are subject to their own thermal landscapes and environmental pressures. Although we did not test it, color variation in butterfly larvae can be temperature-induced, and decoupled from the melanization observed in adults as shown in *Danaus plexippus* (Nymphalidae) (Davis et al., 2005). It is also likely that the mechanisms underlying these characteristics in adults and larvae function independently.

The relationship between coloration and environment remains extremely complex, especially in the context of multiple pressures and intersecting gradients that can be explained by abiotic and biotic pressures or phylogenetic origins, that are still hard to decipher (True, 2003; Álamo et al., 2023).

2. Evolution of transparency: a trade-off between multiple selection pressures?

Our work brings unprecedented results with regard to transparency. Chapters 1 and 3 provided the first opportunity to explore the optical and thermal properties of the wings in multiple butterfly species, including many transparent ones. We clearly demonstrated that transparent patches absorption properties are poor compared to those of opaque patches, whatever the range considered, from UV to NIR. Their thermal properties are therefore greatly impacted, and the heating ability of these patches was much lower when exposed to light than any other color. This makes transparent wings a poor heat absorption feature, and it is hard to imagine them playing a predominant role in thermoregulatory processes in these butterflies. Plus, when we studied the developmental consequences of temperature in *Methona confusa*, we found no change in the optical properties of transparent patches in adults as a function of temperature conditions during development.

It is possible that the plasticity of transparent zones suffers from an ontogenetic limitation, as it is different from a simple change in coloration. This is probably more of a micro- and nano-structural matter. Transparency in butterflies is achieved by a modification in the structure of the scales, the pigment-containing structures on the surface of the wing membrane. In transparent patches, and depending on the taxon, the scales can be absent, thinner, of low density or even pigmentless (Gomez et al., 2021b). Nanostructures on the surface of the membrane also play a role in light transmission, their presence providing the wing anti-reflective properties (Yoshida et al., 1997; Pinna et al., 2021). Pomerantz et al. (2021) showed that in the early stages of wing development in the Ithomiini species *Greta morgane oto*, scale precursors on the membrane are already reduced in density, and that the cytoskeletal organization of scale cells differs from that of colored flat scales. The effect of development conditions on these structures remains to be studied.

More than one mechanism can simultaneously affect the selection for melanism, and more generally, colorations, in ectotherms. Wing colorations and patterns evolve under multiple simultaneous ecological pressures, which overlap or oppose each other, whether biotic or abiotic (Postema et al., 2022). In the first chapter, we discussed the possible interference of biotic selection pressures on butterfly wings, which would interfere with the optimization of climate adaptation traits. Numerous examples of multiple trade-offs have been documented in butterflies, be it between aposematism, thermoregulation and immunoregulation in *Parasemia plantaginis* (Erebidae) (Lindstedt et al., 2009; Hegna et al., 2013), camouflage and mimicry in *Heliconius sp.* (Nymphalidae) (Bybee et al., 2012), camouflage and intraspecific signaling in *Curetis acuta* (Lycaenidae) (Wilts et al., 2013).

In Ithomiini, wing transparency has been shown to enhance crypsis. The more transparent the wings, the less they are detected by naïve predators (Arias et al., 2019). On the other hand, transparent patches have been showed to be part of the aposematic pattern signal,

because the way the light is reflected by these patches is a convergent trait within the mimicry rings (Pinna et al., 2021). In fact, there is most likely a trade-off between this aposematic signal and the crypsis strategy. McClure et al. (2019) has shown that the more transparent species are also the most unpalatable to birds, likely making them more memorable to experienced predators despite their cryptic color patterns. In addition, predation pressure likely vary with altitude (Roslin et al., 2017). Fiedler and Brehm (2021) found that in Artctiinae, a clade of moths, cryptic colorations were more common at higher altitudes whereas aposematic colorations were more common a lower altitudes. They suggested that this was driven by the altitudinal distribution of predation pressure, assuming that warning patterns are less effective in a context of relaxed predation. Thermoregulation is not the only function to suffer from this trade-off. Hydrophobicity is another example. Indeed, optical transparency and water repellency have been found to be negatively correlated : the most optically transparent wings are the least water repellent, because of their reduced or absent microstructures, while hydrophobicity increases as the surface on the wing membrane gets more complex, with nano and microstructures (Gomez et al., 2021a). Finally, the genes involved in melanization in insects probably affect more than one trait, and are intrinsically linked to other vital functions, from fertility to pathogen resistance (True, 2003). We can conclude that the optical and thermal properties of the wings in Ithomiini probably contributes little to thermoregulation, and that the thermal selection pressure on wing features is likely lower than those on other aspects of their ecology.

To better understand how transparency is affected by thermal constraints, it would be worth exploring whether the results found in Ithomiini apply to other clades of clearwing species. It is difficult to find a system comparable to that studied here. Species of other taxa involved in the transparent ithomiine mimicry rings are likely subject to the same selection pressures on wing pattern convergence, like the Dioptini (Notodontidae) and Pericopina (Erebidae). The neotropical tribe Haeterini (Nymphalidae) is probably the most striking: these butterflies that fly in close proximity to the forest floor include perfectly opaque dark species, and others that are almost totally transparent (Fig 1), which a priori have a similar behavior and microhabitat, and fly at altitudes of around 1000 m, but of which a transparent species, *Pseudhaetera hypaesia* live at altitudes of up to 2000 m (informations from the Sangay national park inventory, Ecuador <https://www.sangay.eu/>). Another promising system for studying the evolution of transparency in butterflies is the Acreaeini tribe (Nymphalidae), which are aposematic butterflies. A large proportion of species live in mountainous systems in tropical Africa, and, like the Ithomiini, a number of species display transparency on their wings (Fig 1). Some transparent *Acreae* can be found up to 2500m.

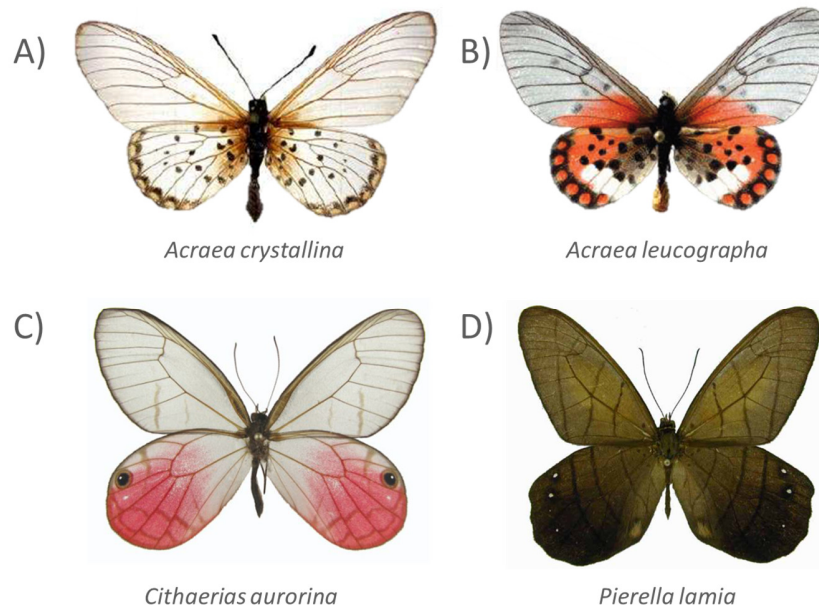


Figure 1. Two interesting systems of butterfly clade for the study of transparency in tropical mountain systems. A) and B) two species of the genus *Acraea* (Nymphalidae), of which a large number of species are native to tropical Africa. Many montane species are transparent. C) and D) two species of the neotropical tribe Haeterini (Nymphalidae) found in the Ecuadorian mountains. (Photos : Andrew Neild).

3. Adaptation and plasticity of physiological response to temperature in Ithomiini butterflies

The results of Chapters 2 and 3 allow us to explore the causes of local thermal adaptation in ithomiine butterfly communities of closely related species. In the Andes, we found a difference in thermal strategy, as well as climate-related thermal tolerance, between high and low altitude populations. Indeed, the low altitude species were more clearly thermoconforming. It remains unclear whether the high altitude species have a specific thermoregulatory strategy, as the relationship between their thoracic temperature and air temperature follows a pattern that can be interpreted either as a very poor ability to thermoregulate (Bladon et al., 2020), as neutral (Hailey and Coulson, 1996), or as a way to boost thoracic temperature as the conditions gets closer to their optimum (Schreiner et al., 2020). Then, high-altitude species in the Andes are more resistant to cold stress, but more sensitive to heat stress than warm-lowland species. The thermal tolerance tests of the two species studied in chapter 3 shows that there is a plastic potential for cold tolerance, as adults that have been reared in colder temperature resist better to cold stress than adults that experienced warmer developmental conditions. Although this does not appear to be the case for heat, it does demonstrate a degree of local acclimation in

the response of individuals to temperature. These results suggest that the adaptive response to temperature is likely due to both directional genetic selection and thermally induced plastic potential. Common garden rearing of species from different altitudes could enable us to assess the contribution of these causes and better understand the effect of environmental filtering on these lineages (Karl et al., 2008). Such a study was initially planned in the Ecuadorian Andes, but made impossible by the covid outbreak. In a similar research project, but focusing at the intra-specific level, Montejo-Kovacevich et al. (2020) has shown that *Heliconius erato* (Nymphalidae) from high-altitude populations in the Andes have a lower heat stress tolerance than low-altitude populations. After rearing in a common garden, the difference between the populations is reduced, indicating plasticity in individual response. Overall, our analyses, which take into account the relationship between species, show an adaptation of the physiological capacities of ithomiines to their environment, in contrast to wing traits. Their observed response is probably the result of genetic, along with plastic adaptation, at least for cold tolerance.

In chapter 3, we showed that life history traits are sensitive to rearing temperature. Individuals reared in warm conditions have a faster developmental rate, but reach a smaller size than those reared in cold conditions, in accordance with the statement of the temperature-size rule, which predicts that the thermal reaction norm for the adult size has a negative slope with developmental temperature (Atkinson, 1994; Angilletta and Dunham, 2003), which, even if its origins are poorly understood, is supported by numerous empirical observations (Gibert et al., 2004; Kingsolver and Huey, 2008). Body size is an important parameter in the thermoregulation capacities of insects. It is greater in colder habitats and provides a more stable body temperature (Bishop et al., 2016; Oyen et al., 2016; Xing et al., 2018). These fitness-related responses are not necessarily adaptive, and the effects detected are sometimes as beneficial as they are detrimental. Achieving and maintaining a large size also entails significant costs and risks, and a long development time may lead to increased energy requirements, delayed sexual maturation and reduced fecundity (Kingsolver and Huey, 2008).

In our work, we have not explored the thermal tolerance of immature stages to acute cold and heat stress. Nevertheless, the larval survival probability at the highest temperature treatment was significantly lower than any other treatment in both *M. confusa* and *T. harmonia*, which demonstrates a high stress induced by high temperatures on the most fragile stages. In mayflies, thermal tolerances are specific to a given stage of development, which ultimately allows for a wide variety of conditions over the entire life cycle (Uno and Stillman, 2020). Thermal conditions experienced by crane fly larvae and adults suggest that high temperatures experienced by immature stages have a negative impact on adult life-history traits such as dispersal capability (Jourdan et al., 2019, 2021).

4. Climate change and resilience potential in tropical butterflies

The decline in insect populations is affecting all ecosystems in aquatic, terrestrial, polar to tropical environments. Its effects are already visible. For instance the biomass of flying insects has already fallen by 76% between 1990 and 2017 in a long-term monitoring in Germany (Hallmann et al., 2017). In a review published in 2019 on the deterioration of entomofauna, it is shown that at this rate 40% of species will have disappeared in the coming decades, with the most fragile orders being Lepidoptera, Hymenoptera and Coleoptera (Sánchez-Bayo and Wyckhuys, 2019). It is specified that while the decline in temperate regions is mainly due to habitat degradation, pollution, invasive species, agriculture and the use of pesticides, climate change appears to be the predominant factor in the tropics. Moreover, while tolerance to cold shows a plastic and likely adaptive response to the rearing temperature, this is not the case for heat tolerance. A review on vertebrate and invertebrate ectotherm taxa reported that phenotypic plasticity is asymmetrical between the upper and lower limits, as upper critical temperature have a limited acclimation response (Gunderson and Stillman, 2015). Although it is not clear why, the narrow upper thermal tolerance may be due to the thermal sensitivity of macromolecular structures in metazoans to hot temperature (Pörtner, 2002; Robertson, 2004), or behavioral buffering could possibly be more effective to lower than to rise body temperature in ectotherms, such as hypothesized by Gunderson and Stillman (2015).

Forecasting the response of insects to extreme temperature events is difficult. They are highly unpredictable in time and space, which can hamper the insects' adaptive response. Numerous studies have now highlighted the role of plasticity in the development of phenotypic responses to contemporary disturbances such as climate change i.e. (Ghalambor et al., 2007; Charmantier et al., 2008; Buckley and Kingsolver, 2019). Our work gives some hints as to the adaptability of species to climate variations. We detected a difference in the thermal tolerance of Ecuadorian species along the elevation gradient, with high-altitude species showing less sensitivity to cold than low-altitude species, but very little resistance to warm thermal stress.

Finally, we found larval stages, from eggs to around fourth instar of *Methona confusa* and *Tithorea harmonia* to be particularly sensitive to temperature, with a lower survival probability at the warmer temperature treatment. Our plasticity experiments focused on a constant exposure during egg, larval and pupal development to a given temperature. While this gives us reliable indication of the plastic properties of traits linked to thermal tolerance (Fischer et al., 2011), it is likely that in nature disturbances are inconstant over time and unpredictable environmental variability limits adaptive responses and may affect immature stage mortality. All these results raise questions as to the resilience of these butterflies to rising temperatures.

The ability to buffer sub-optimal temperature may mitigate the effects of variations in temperature conditions. We did not find a clear buffering ability in the Andean populations. However the unusual pattern of thermoregulatory capabilities of high-altitude species, where

butterflies increase their temperature more in warmer ambient temperatures, need to be studied in greater detail. More generally, the buffering capacity of terrestrial ectotherms is being undermined. In most environments, rising temperatures are bringing individuals closer to their maximum thermal critical temperature, leaving them with virtually no thermal buffer margin. This margin is predicted to disappear by the end of the century for tropical species (Kingsolver et al., 2013). By living closer to their upper thermal limits, they should have greater thermal sensitivity and less room to manoeuvre in the face of global warming (Deutsch et al., 2008; Perez et al., 2016) (Fig 2).

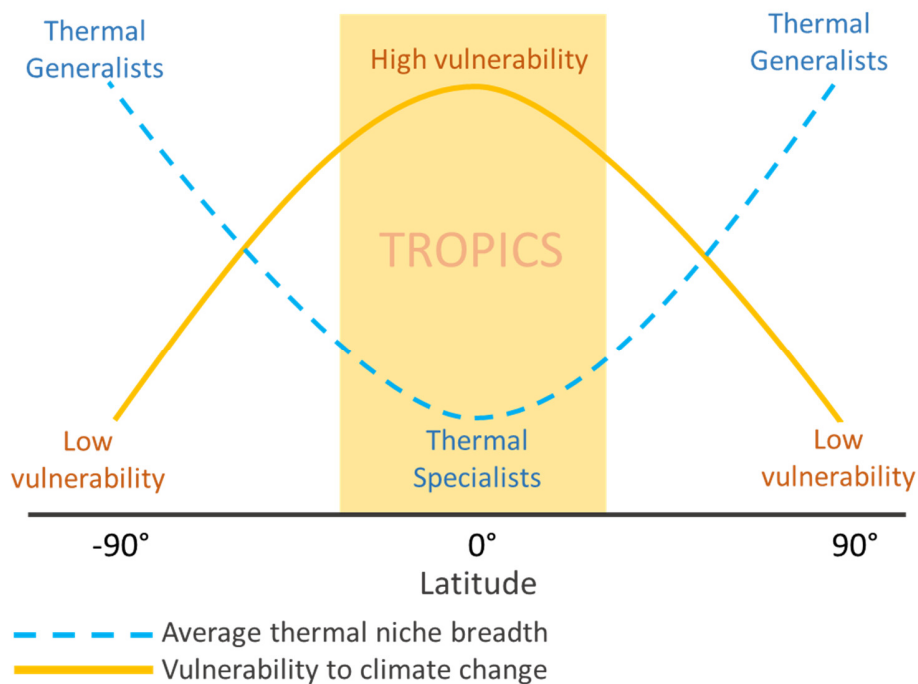


Figure 2. Adapted from Perez et al.(2016). The thermal niche of tropical species, i.e. those living at low latitudes, is narrower than those living at high latitudes. Exposed to less climatic variability, they tend to be thermal specialists, but this implies a risk of vulnerability in the event of disturbances such as those caused by climate change.

Studies suggest that ectotherms thermal tolerance breadths are driven by the cold tolerance, which increases toward the poles and with elevation, while high-temperature tolerance varies little with geography (Sunday et al., 2011, 2019). In high altitude, habitats are characterized by periods of high insulation which result in high temperatures. Coupled with the drop in temperature with elevation, species inhabiting high-altitude are exposed to a wider diurnal and annual range of temperature than in low altitude. Therefore, high altitude species have in general wider thermal breadths than low altitude ones (Sunday et al., 2019). Although we also denoted a wider range of thermal variations in the high-altitude study site in the Andes,

ithomiine community seemed far more sensitive to heat than the species inhabiting the lowlands. While we do not know the critical upper and lower temperatures of the species studied, the cold temperatures experienced in the tropical jungle do not seem to present any significant risk of body damage, unlike heat stress, which resulted in substantial mortality after our tests. While adaptive tolerance adjustments could reduce the impact of climate change, we observe that in mountain ecosystems, the tendency of most organisms is to shift their range upwards, following the shift in favorable climatic conditions (McCain and Garfinkel, 2021).

Adaptation to altitude involves a whole range of environmental -biotic and abiotic-factors specific to each system. It is clear that the response and resilience of species to rising temperatures is balanced by the selective pressures associated with these other parameters. For instance, the geographical shift towards thermally suitable environments may also be greatly hindered by the oxygen limitation in altitude (Jacobsen, 2020). Furthermore, the altitudinal zonation of species can depend on interspecific communication, mutualistic mimicry, host-plant interaction and behavioral interference (Willmott and Mallet, 2004; Chazot et al., 2014; Grether and Okamoto, 2022). The Ithomiini are all part of mimicry rings, and are vertically segregated by flight height, from the understory to the canopy (Beccaloni, 1997; Elias et al., 2008; Willmott et al., 2017). As the response to global change varies between species, the whole structure of communities can be affected. Interacting species may have different thermal tolerances and dispersal capacities, and some species will have more aptitude than others for tracking their niche and shifting their range, leading to the loss or the formation of biotic interactions (Sheldon et al., 2011). Among them, we can cite the modification of plant-pollinator interactions: the changing identity of pollinator species and the plants to which they are exposed, in a context of geographical shifting, is leading to a decline in the reproductive success of plants in alpine environments (Richman et al., 2020). The reduction of the co-occurrence of herbivores with their host plants, (Cerasoli et al., 2019; Poloni et al., 2022) could specifically endanger species that have no alternative host plants, which is the case for most ithomiine species (Willmott and Mallet, 2004). Likewise, the divergence of thermal tolerance between predator and prey can change the structure of their interaction (Rosenblatt et al., 2019). Shifts can also be temporal, and as phenological responses are disrupted, the food web is weakened (Harvey et al., 2020). In some counterexamples, these modifications are accompanied by cascading effects of facilitation: transplantation of grasshoppers which have developed new herbivory interactions, reduced the biomass of native plants and facilitating the establishment of new species in the Alps (Descombes et al., 2020). Tropical mountain ecosystems are particularly at risk of community disassembly. Because species elevational ranges are narrower at these latitudes, the risk of mismatch between interacting species is consequently higher (Sheldon et al., 2011). Such food web collapses are already visible. In the primary forests of Puerto Rico, the fall in arthropod biomass - up to 98% for some taxa over several years of monitoring - has been accompanied by a drop in bird, lizard and amphibian populations (Lister and Garcia, 2018).

Taking refuge in the shade, standing in the sun, or adapting the hours of activity during the day are common ways used by ectotherms to regulate their temperature (Lambrinos and

Kleier, 2003; Barton et al., 2014). We found no evidence of potential thermal behavior of adult Ithomiini to buffer their temperature. Temperatures offered by microhabitats can help to relieve the pressure of global climate variations (Pincebourde and Suppo, 2016). Combined with genetic variance and plasticity of the thermal limits, microhabitat choice may explain the ability to colonize different altitudes in ecotherms, as suggested for *Heliconius* neotropical butterflies (Montejo-Kovacevich et al., 2020). In rainforests, the understory is more stable in temperature than the canopy in rainforests (Kaspari et al., 2014) and the average difference between understory and canopy can be more than 2°C in Ecuador (Montejo-Kovacevich et al., 2020). Plus, soil and vegetation reduce the maximum temperature and offer efficient thermal haven (Scheffers et al., 2013). At the interspecific level, there is a variation of more than 10°C in the upper thermal limit in a community of Panamanian ants. This limit is higher for canopy species, which are more exposed to the sun. This makes canopy a more variable environment in temperature conditions than the ground level, and is therefore more likely to host thermal generalists (Kaspari et al., 2014). There is a lack of information on the use of microhabitat by tropical butterflies to buffer their temperature. We already know that Ithomiini species and mimicry rings are segregated by flight height in the forest, and an explanation for this layering likely involves a multitude of adaptations (Willmott et al., 2017). However, microclimatic refuges exist on several scales, and very fine microclimates on a plant or a single leaf expose individuals to a specific range of temperatures, sometimes explaining the variations in the thermal limits of insects living within the same macrohabitat (Pincebourde and Suppo, 2016). This is particularly interesting for the immature stages of butterflies.

Nothing is yet known about the thermal niche of Ithomiini larvae *in natura*, or their behavior on the host plant during periods of strong temperature variation. Yet, it is more than likely that the thermal landscape experienced by larvae and imagoes are very different. That said, microhabitat choice and behavioral adjustment in general as a means of escaping from the variations in climatic conditions can reduce selection pressures, and thus hinder evolution (Muñoz, 2021). Rearing experiments which would focus on characterizing behavioral adjustment of tropical butterflies would be useful to understand their buffering margin.

5. Overview of another mountain system: the alpine butterfly community

Context

Temperate mountains are subject to greater seasonality and a wider daily temperature range than tropical mountains (Addo-Bediako et al., 2000). In consequence, adaptive traits should respond to more variable conditions, and vary with elevation, which influences thermal aptitude. For instance, some species of butterflies of the genus *Coenonympha* (Nymphalidae) at higher elevations have a higher warming-up rate, which provides them a higher body

temperature at takeoff (Nève and Després, 2020). Alpine butterflies have much wider altitudinal niches than in the Andean mountains. The vast majority of species in the French Alps are thermal generalists, while specialist species are restricted to the extremes low (<500m) and high (>2000m) altitudes (Gallou et al., 2017). Between these two limits, species turnover is low, and roughly the same species are found along the elevation gradient.

When I began the study of Ecuadorian butterfly communities in the Andes, presented in chapter 2, my work had to be suddenly interrupted by the coronavirus outbreak. Unable to travel to the field for 2 years to collect data, we initiated a parallel project on alpine butterfly communities, which is still ongoing today. I had to implement methods and protocols to measure thermal tolerance in butterflies, and I was able to calibrate the techniques and equipment used for the study in Ecuador and later in Panama (chapters 2 and 3). The aim of the project is to study the complementarity between the physical characteristics of butterfly wings and bodies (colors, radiative properties, structures) and the biology of each species (behavior, period of activity during the day, take-off temperatures). By comparing the thermal abilities of very different clade species exposed to a wide range of climatic conditions, this project aim to provide essential elements for understanding what had shaped the traits involved in the thermal adaptation of alpine communities. We focused on the most common Nymphalid and Pierid species, and tried to collect species with a wide range of colorations, spanning from a high degree of darkness such as in the genus *Erebia*, to very light-colored and partly translucent wings like in the genus *Aporia*.

During this field session, I was able to collect substantial data on the thermal tolerance of alpine butterflies of low and high-altitude communities, in order to test whether living individuals sharing the same altitudinal niche have similar thermal tolerances. I measured individuals' tolerances to cold and hot stress, and analyzed the relationship with the altitude of their communities. I tested the prediction that individuals from high-altitude communities should be more resistant to cold and more sensitive to heat than low-altitude ones, reflecting thermal adaptation to climate.

Material and methods

We sampled butterflies in the following departments: Isère, Hautes-Alpes and Alpes de haute Provence (Fig 2). We focused on altitudinal gradients, and in each locality visited, we carried out a collection session at high (~2000m), and low altitude (~1000m). To compare butterflies temperature tolerance, we performed static assays to tests cold and heat tolerance of the individuals, with the same material and protocols described in chapter 2, but with differences for the tested temperatures. For the cold resistance test, individuals were placed in refrigerated jars at 3°C, and 47.5°C for the heat resistance test. We tested the relationship between individuals' thermal tolerance and their altitudinal community, as well as their weight and sex, via mixed models with the function `lme` from the R package `nlme` (Pinheiro et al., 2023) with species as random effect. As in chapter 2, to disentangle the effect of shared ancestry from

adaptation, we also performed Bayesian mixed models correcting for phylogeny with the R package MCMCglmm (Hadfield, 2010) using the recently published phylogeny of all 496 European species (Wiemers et al., 2020).

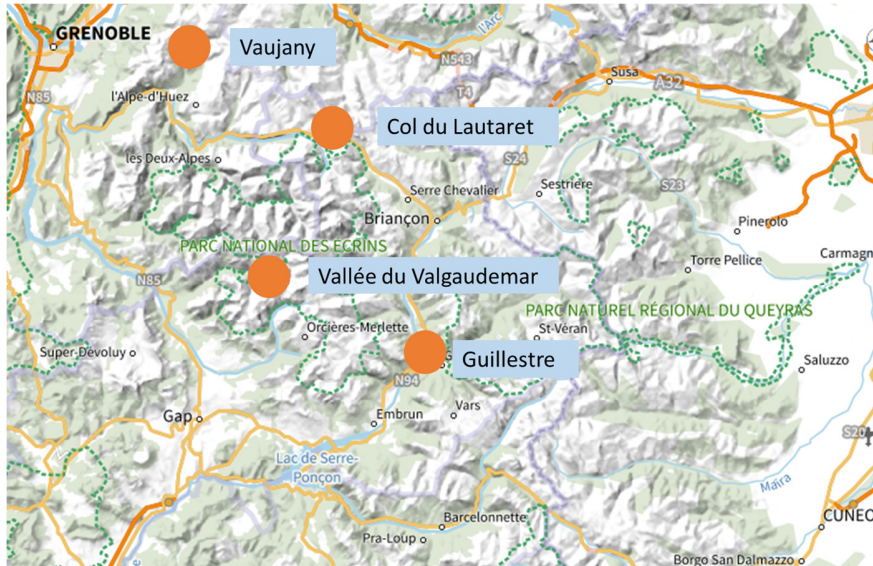


Figure 3. Map of sites visited during the collection campaign in the Alps, in the Ecrins National Park and surrounding areas. At each site, two butterfly communities were sampled, one at high altitude (around 2000 m) and one at low altitude (around 1000 m). We thus had a total of 8 collection sites, four highlands and four lowlands.

Results and conclusions

We carried out thermal tolerance tests on 474 specimens of 29 common species, belonging to the Nymphalidae and Pieridae families. When exploring the thermal tolerance of Alpine species, we found no significant difference between communities in their recovery time after cold stress, nor in heat resistance, in our mixed model (Appendix Tables S1 – S2). In the cold shock, we detected that individuals from the low-altitude community were significantly more resistant to knock-out, according to mixed models, than high-altitude individuals (Appendix Table S3).

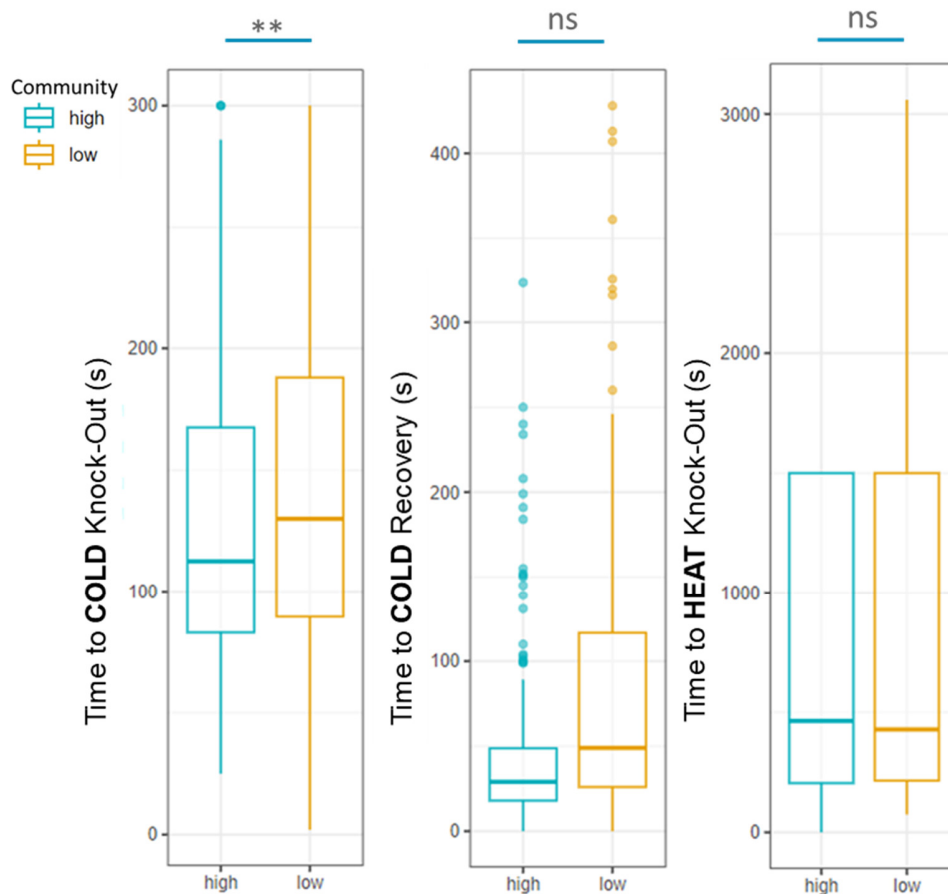


Figure 4. Thermal tolerance comparison between the highland and lowland communities of alpine butterflies. From left to right: Time until knock-out in seconds of the highland and lowland communities during the cold shock experiment; time until recovery in seconds of the highland and lowland communities during the cold shock experiment (3 °C); and time until knock-out in seconds of the highland and lowland communities during the heat shock experiment (47.5 °C). The significance of the difference between the two groups is based on the predictions of the lme models, with a p-value set at 0.05. However the significance of the effect for cold knock-out cease when corrected for phylogeny, in the Bayesian model (see Tables S1 to S4, Fig S1).

In addition, heavier individuals resisted longer. However, all these effects disappear when phylogeny was taken into account in the analyses (Appendix Table S4). Indeed, phylogenetic signal measurement, using Blomberg's K method (Münkemüller et al., 2012), where K values closer to zero correspond to a random or convergent pattern of evolution, and high K values indicate strong phylogenetic signal, showed that there was a significant phylogenetic signal for cold knockout resistance ($K=0.432$, $p < 0.001$) as well as for heat resistance ($K=0.285$, $p < 0.019$), but it was not significant for cold recovery ($K=0.2434275$, $p < 0.074$).

Overall, thermal tolerance of alpine species does not seem to vary much along the altitudinal gradient, and the differences observed appear to be explained by phylogeny, and relatively conserved within lineages, as we detected a phylogenetic effect in thermal responses. In the field, we sampled virtually identical communities between high and low altitudes, with little species turnover. Temperate species have greater dispersal capacities, overlapping high and low altitude conditions throughout the seasons or day, as temperature conditions vary considerably throughout the day and throughout the year (Ghalambor, 2006). This translates into wider thermal niches, and in this example, we can see that the hot and cold test temperatures, calibrated to detect an individual response during the trial (3°C and 47.5°C), are more extreme than for tropical species (5°C and 39°C).

These results are inspiring for future research, as it reveals the variability in response that exists between species, something we had not observed in the Andes, as the comparison was made within the same tribe, with relatively little phylogenetic variation. The greatest divergence within the Ithomiini tribe is 24.6 million years (Chazot et al., 2019), whereas the Pieridae and Nymphalidae studied here are separated by 101 million years, and 82 million years just within the alpine Nymphalidae (Wiemers et al., 2020). In the Alps, the phylogenetic component seems to be of prime importance in the response of species to temperature, and explains a large part of the results. Each lineage appears to have its own way of coping with thermal stress. In the field, alpine species showed a wide range of trait associations. For example, highly melanized species such as *Maniola jurtina* or *Erebia alberganus* (Nymphalidae: Satyrinae) are better able to withstand heat, and have proven to be the most thermophilic, while lighter species such as *Aporia crataegi* or *Anthocharis cardamines* (Pieridae) proved particularly sensitive to heat (Figure S1). The optimum locomotor temperature is likely higher for the darker species, and raises the question of whether the color pattern has been adapted to this optimum, thereby maximizing the butterfly's thermoregulatory capacities. Altitude has a marked effect on other traits, such as phenology and the number of generations per year, which is closely linked to the annual life cycle of host plants (Hodkinson, 2005). In these particularly harsh climates, physiological mechanisms are involved in cold tolerance, such as the presence of cryoprotectants (Vrba et al., 2017) or the production of heat shock protein for protection against high temperatures (Gonzalez et al., 2022).

This preliminary study, which will be completed with species in other butterfly families, along with my thesis project on Ithomiini, shows that thermal melanism is not so obvious. The expectation of thermal melanism is undoubtedly too strong compared with the reality of the balance between neutral processes (e. g., Brownian evolution along the phylogeny), constraints and selective pressures that collectively influence color variations in butterflies (True, 2003; Dufour et al., 2018; Fiedler and Brehm, 2021; Postema et al., 2022). Indeed, many studies assessing color variation along climatic gradients found a wide dispersion of the color and reflectance measurements according to geography i.e.(Munro et al., 2019; Kang et al., 2021; Álamo et al., 2023), which may reflect the complex interplay between those multiple evolutionary processes. Spectrophotometric measurements, as well as infrared imaging, will

enable us to learn more about the thermal properties of alpine wings, and whether they vary along the altitude gradient and the phylogeny, in the continuation of this project.

6. Conclusion

Our work has highlighted the importance of a multi-disciplinary and multi-scale approach to the study of thermal adaptation. This applies not only to butterflies, but to all types of organisms, because thermoregulation is a complex process and climatic adaptation is the result of an intricate interweaving of traits. The aim of our study was to be integrative and at the interface between physics and ecology. Our work has provided interesting propositions in the way of using precise imaging tools, in particular spectrophotometry and thermal imaging cameras, to answer a biological question. We have highlighted a number of counter-intuitive results, such as the distribution of the coloration pattern and wing properties with elevation, or the absence of thermally induced plasticity of wing traits, probably constrained by other selection pressures. Yet, we also highlighted the strong environmental response in thermal tolerance in ithomiine species and communities.

The study of wing transparency has provided novel insights into the thermal implication of such a peculiar trait in clearwing butterflies. For the first time, thermal and optical properties of transparent patches and their response to developmental temperature, have been described in detail. Our study complement our knowledge of the ecology of these butterflies and the trade-offs involved with transparency (Chazot et al., 2014; Arias et al., 2019; McClure et al., 2019; Doré et al., 2022), thus providing a better understanding of selection pressures that have shaped the traits observed in these very unique clearwing butterflies and their possible implication with regard to climate variations, in the past and in the future.

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Appendix to General Discussion

Table S1. Retained linear mixed model of the relationship between time until recovery of the alpine butterflies during the cold shock experiment (3°C) and the altitudinal community, after backward selection of fixed parameters based on the p-value, with species as random parameter. Significant fixed effect estimates are indicated in bold, associated with a p-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model. Here, the null model was retained.

Dependent variable	Effect	Value	Std.Error	DF	T.value	Estimated std Error	P-value
Time to COLD recovery	(Intercept)	61.413	8.162	437.000	7.525	61.41 ± 8.16	<0.001***
	Other fixed parameters not retained : community, weight, sex, mean temperature in the jar, standard deviation of the temperature in the jar						

Table S2. Retained linear mixed model of the relationship between time until recovery of the alpine butterflies during the heat shock experiment (47.5°C) and the altitudinal community, after backward selection of fixed parameters based on the p-value, with species as random parameter. Significant fixed effects are indicated in bold, associated with a p-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model. Here, the null model was retained.

Dependent variable	Effect	Value	Std.Error	DF	T.value	Estimated std Error	p-value
Time to HEAT knock-out	(Intercept)	731.381	79.204	407.000	9.234	731.38 ± 79.	<0.001***
	Other fixed parameters not retained : community, weight, sex, mean temperature in the jar, standard deviation of the temperature in the jar						

Table S3. Retained linear mixed model of the relationship between time until knock-out of the alpine butterflies during the cold shock experiment (3°C) and the altitudinal community, after backward selection of fixed parameters based on the p-value, with species as random parameter. Significant fixed effects are indicated in bold, associated with a p-value (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Effect	Value	Std.Error	DF	T.value	Estimated Error	std	p-value
Time to COLD Knock-Out	(Intercept)	21.85	17.97	403.00	1.22	21.85 ± 17.97		0.225
	Community High>Low	-23.42	7.22	403.00	3.24	-23.42 ± 7.22		0.001**
	Weight	0.19	0.02	403.00	7.73	0.19 ± 0.02		<0.001***
	Mean temperature in the jar	29.34	5.85	403.00	5.02	29.34 ± 5.85		<0.001***
Other fixed parameters not retained : sex, standard deviation of the temperature in the jar								

Table S4. Retained Bayesian mixed model correcting for phylogenetic relatedness of the relationship between time until knock-out of the alpine butterflies during the cold shock experiment (3°C) and the altitudinal community, after backward selection of fixed parameters based on Bayesian p-value criteria. (*P < 0.05; **P < 0.01; ***P < 0.001). The factors for which the parameters were not significant are listed below the model.

Dependent variable	Parameter	Posterior mean	Lower 95 % Confidence Interval	Upper 95 % Confidence Interval	Effective size	Bayesian p-value
Time to COLD Knock-Out	(Intercept)	69.326	6.073	131.689	9054.106	0.039*
	phylogenetic variance	1808.037	0.000	5265.375	129.634	
	Species identity	427.965	0.000	1534.640	114.567	
	residual variance	4452.771	3844.354	5069.644	19500.000	
Other fixed parameters not retained: community, weight, sex, mean temperature in the jar, standard deviation of the temperature in the jar						

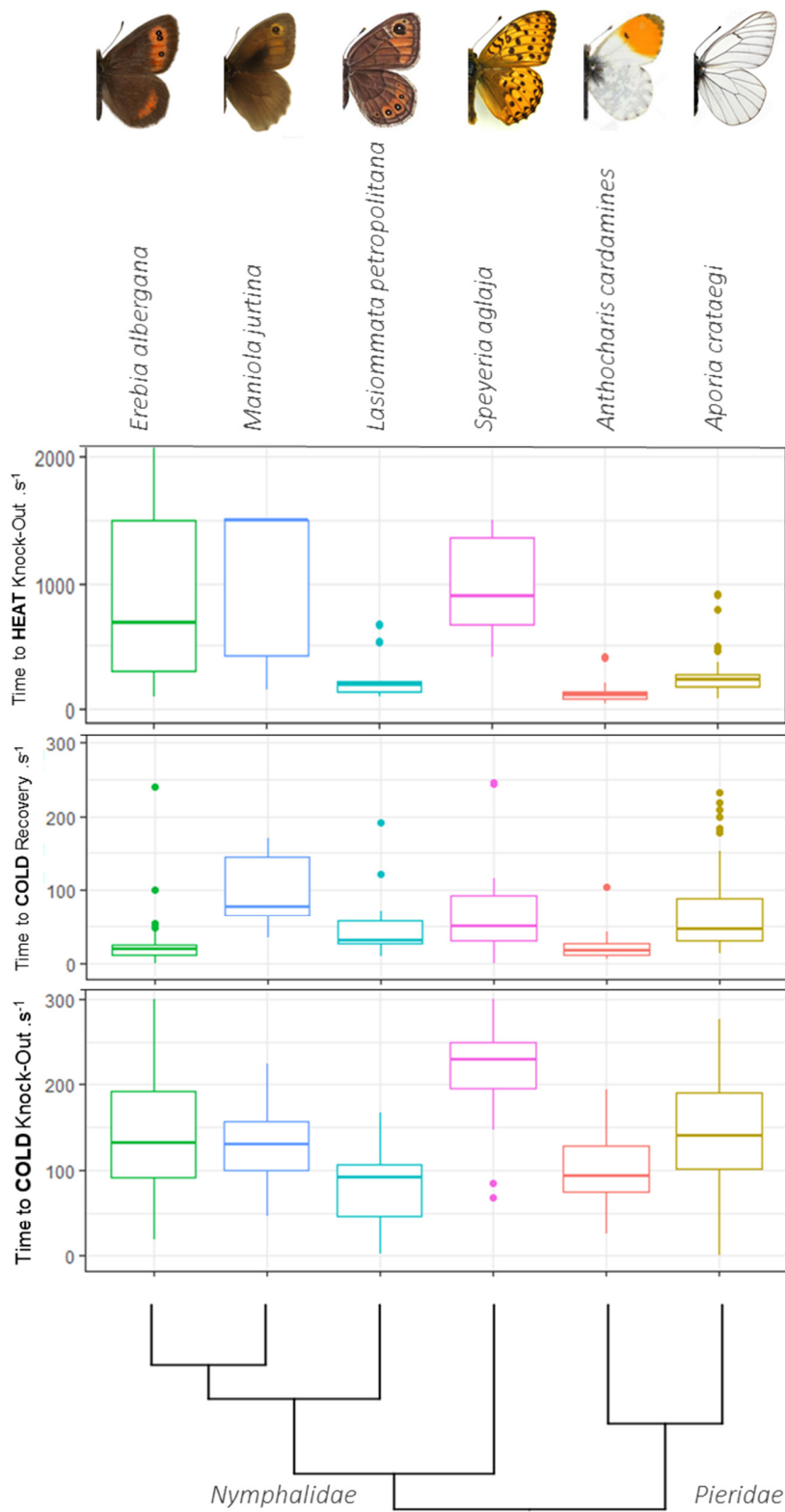


Figure S1. Legend next page.

Figure S1. Thermal stress tolerance as measured via cold knock-out time, cold recovery time and heat knock-out time, for five species of Nymphalidae and Pieridae Alpine butterflies. In the Alps, differences between species seem to be linked more to phylogeny than to elevation.

Résumé substantiel

L'activité des ectothermes est limitée par les conditions environnementales, notamment par la température ambiante. Leur capacité à ajuster leur température corporelle et à tolérer les situations de stress thermique est le résultat de mécanismes morphologiques, physiologiques et comportementaux. De plus, la température a un effet documenté sur leur développement et peut induire des effets plastiques sur leur morphologie et leurs performances thermiques. Contrairement à la plupart des papillons qui arborent des ailes colorées et une grande variété de motifs, certaines espèces présentent des ailes transparentes à divers degrés, pour lesquelles l'absorption de la lumière est limitée. La transparence des ailes, qui implique une réduction de la pigmentation, ou une modification structurelle des écailles et de leur densité, soulève des questions concernant les coûts potentiels pour les fonctions vitales, telles que la thermorégulation, en particulier le long des gradients climatiques. En outre, de nombreux papillons néotropicaux à ailes transparentes se trouvent à haute altitude, dans des climats plus froids, où l'on s'attend à ce que les espèces arborent des couleurs plus sombres. L'hypothèse du mélanisme thermique postule que les colorations plus foncées devraient être favorisées dans les environnements plus froids, ce qui leur permet d'absorber plus de rayonnement et de maintenir une température corporelle compatible avec leur activité. Cette prédiction s'étend au domaine du proche infrarouge (NIR), qui représente une grande partie du rayonnement solaire atteignant la surface de la terre. Chez les papillons, l'effet de la température de développement peut également influencer les caractéristiques des ailes liées aux capacités d'absorption de la chaleur, telles que le degré de mélanisation. La compréhension des processus complexes impliqués dans l'adaptation thermique de ces papillons nécessite une approche interdisciplinaire, combinant l'étude du phénomène physique de l'absorption de la lumière par les ailes, associée à l'exploration des besoins thermiques, de la tolérance et du comportement qui contribuent à la thermorégulation. Dans ma thèse, je me suis concentrée sur la tribu néotropicale des papillons Ithomiini (Nymphalidae), qui comprend environ 400 espèces mimétiques, réparties entre les plaines de basse altitude au climat chaud et les hautes altitudes, plus froides, dans les Andes. Les Ithomiini sont des papillons intéressants pour étudier le mélanisme thermique puisque la plupart des espèces de ce groupe ont des ailes transparentes ou partiellement transparentes. Les néotropiques et en particulier les Andes sont l'un des points chauds de biodiversité terrestre les plus riches, avec une forte variation altitudinale des facteurs abiotiques et, parallèlement, un fort renouvellement altitudinal des espèces. Dans le chapitre 1, j'ai entrepris une étude comparative des propriétés optiques et thermiques des ailes de papillons Ithomiini, des plus transparentes aux plus opaques, le long du gradient d'élévation, dans un cadre phylogénétique, grâce à une phylogénie publiée en 2019. J'ai testé les implications de la transparence des ailes pour l'absorption de la lumière et les propriétés thermiques chez 42 espèces, et j'ai testé si elles sont conformes à la prédiction de l'hypothèse du mélanisme thermique. Pour cela, j'ai mesuré en spectrophotométrie l'absorption des ailes dans le domaine des ultraviolets (UV), visible et NIR. J'ai quantifié la proportion des ailes occupées par une coloration foncée. J'ai également filmé les ailes en imagerie thermique pour évaluer leur capacité à s'élever en température. J'ai constaté que les ailes transparentes sont moins efficaces que les ailes opaques pour absorber la lumière dans les gammes de longueurs d'onde UV, visible et NIR, et qu'elles sont également moins efficaces pour collecter la chaleur.

En outre, la coloration foncée occupe une proportion plus faible de la surface de l'aile à mesure que l'altitude augmente, et les espèces d'Ithomiini sont plus transparentes à haute altitude, où les conditions climatiques sont plus froides, ce qui va à l'encontre de la prédiction de l'hypothèse du mélanisme thermique. De plus, les propriétés optiques et thermiques des ailes ne sont pas prédites par les conditions climatiques. La coloration des ailes des Ithomiini ne semble donc pas contribuer à l'adaptation à l'altitude, ce qui s'écarte des études faisant état d'une corrélation entre le mélanisme et le climat chez les insectes. Je discute de ces résultats surprenants à la lumière d'études récentes suggérant que des facteurs autres que l'adaptation thermique, tels que la pression de prédation, la physiologie ou le comportement, ont pu être à l'origine de l'évolution des motifs alaires chez ces papillons. Dans le chapitre 2, j'ai évalué la capacité des espèces de deux communautés d'Ithomiini de basse et de haute altitude dans les Andes équatoriennes, à ajuster leur température, ainsi que leur tolérance thermique à des températures extrêmes. J'ai ainsi mesuré la température corporelle de papillons d'une soixantaine d'espèces dans leur environnement naturel, et testé en laboratoire leur tolérance à une exposition au froid (5°C) et au chaud (39°C). Les espèces des basses altitudes sont généralement thermoconformistes, c'est-à-dire que leur température corporelle diffère peu de celle de l'environnement. En revanche, les espèces des hautes altitudes présentent un schéma d'ajustement de température inhabituel qui pourrait être interprété soit comme une faible capacité de thermorégulation, soit comme un renforcement potentiel de la thermorégulation aux températures élevées. J'ai également constaté que les espèces des hautes altitudes résistaient plus longtemps au froid, mais qu'elles étaient moins résistantes à la chaleur, que les espèces de la communauté des basses altitudes. En prenant en compte la parenté phylogénétique dans les analyses, j'ai montré que les variations observées sont dues à l'adaptation à l'environnement et pas seulement à une ascendance partagée. Les résultats mettent en évidence la sensibilité thermique des populations vivant en haute altitude et soulèvent des inquiétudes quant aux impacts du changement climatique sur les montagnes néotropicales, qui sont parmi les régions du monde qui se réchauffent le plus rapidement. Dans un troisième chapitre, j'ai élevé des papillons de deux espèces panaméennes d'Ithomiini, *Methona confusa* et *Tithorea harmonia*, depuis le stade de l'œuf jusqu'à l'âge adulte dans des conditions contrôlées afin de quantifier la plasticité induite par la température des propriétés physiques des ailes et des performances thermiques des papillons. J'ai étudié l'effet de trois traitements thermiques sur le développement et la plasticité des caractères de ces deux espèces. Ainsi, j'ai mesuré les paramètres du développement, tels que le temps de développement, la survie et le poids des larves, ainsi que le poids des imagos, élevés à une température constante de 18°C, 24°C ou 27°C. Puis, j'ai testé l'effet des traitements sur la tolérance au stress thermique des imagos par des tests de résistance au chaud et au froid, et je les ai comparés à ceux des individus des populations sauvages. J'ai enfin évalué la conséquence des traitements de température sur les propriétés optiques des ailes. Le développement larvaire est influencé de manière significative par la température : dans des conditions plus chaudes, la probabilité de survie est plus faible, le temps de développement est plus court, et les larves et imagos sont plus petits. Les propriétés optiques des ailes et le degré de mélanisme ne sont pas significativement différents parmi les papillons exposés aux différents traitements thermiques, ni avec la population sauvage. De même, les zones transparentes sur les ailes de *Methona confusa* n'ont pas été affectées par la température de développement. La tolérance thermique au stress

froid de *Methona confusa* montre une plasticité induite par la température, les individus élevés à 18°C ayant la plus grande tolérance au froid, car ils mettent plus de temps à montrer une perte de locomotion et se rétablissent plus rapidement. La résistance au froid de *Tithorea harmonia* ne montre pas de différences significatives entre les traitements, mais les individus sauvages sont plus résistants. La tolérance à la chaleur n'est pas influencée de manière significative par la température de développement chez ces deux espèces, car je n'ai pas détecté de différence entre les traitements. La température de développement chez ces deux espèces de papillons a donc eu un effet important sur les stades immatures, mais aucun effet sur les propriétés des ailes liées à l'absorption de la lumière. De plus, les résultats suggèrent que la résistance au froid est au moins partiellement due à de la plasticité chez ces espèces. L'absence de variation concernant la résistance à la chaleur et la mortalité élevée des larves lors du traitement à haute température chez les deux espèces soulève des questions sur l'impact du changement climatique sur ces espèces tropicales. Ces résultats confirment l'existence d'une adaptation physiologique aux différentes conditions de température chez les Ithomiini, qu'elle soit génétique ou plastique. Les résultats de la thèse soulèvent des inquiétudes quant à l'impact du changement climatique sur ces populations de papillons, et plus généralement d'insectes tropicaux ayant des niches altitudinales plus étroites que dans les régions tempérées. Le fait que nous n'ayons pas détecté de capacité d'ajustement comportemental met en danger les espèces confrontées à des variations de température inattendues, avec peu de possibilités d'ajuster leur réponse. Ce travail a mis en évidence l'importance d'une approche interdisciplinaire et multi-échelle pour l'étude des adaptations thermiques. Ceci s'applique non seulement aux papillons, mais à tous les types d'organismes, car la thermorégulation est un processus complexe et l'adaptation aux conditions climatiques est le résultat d'une imbrication de plusieurs traits. Le but de cette étude est d'être intégrative et à l'interface entre la physique et l'écologie. Ce travail de thèse a fourni des propositions intéressantes dans la manière d'utiliser des outils d'imagerie précis, en particulier la spectrophotométrie et les caméras thermiques, pour répondre à une question biologique. J'ai pu mettre en évidence un certain nombre de résultats contre-intuitifs, tels que la distribution du motif de coloration et des propriétés des ailes en fonction de l'altitude, ou l'absence de plasticité des caractéristiques des ailes induite par la chaleur, probablement limitée par d'autres pressions de sélection. L'étude de la transparence des ailes a fourni de nouvelles informations sur l'implication thermique d'un caractère aussi particulier chez les animaux. La transparence chez les papillons tropicaux a probablement évolué en réponse à la sélection exercée par la prédation, car elle constitue une stratégie de camouflage efficace, tout en faisant également partie intégrante du signal aposématique. Cependant, j'ai également mis en évidence la forte réponse environnementale en matière de tolérance thermique chez les espèces et les communautés d'Ithomiini. Cette étude complète nos connaissances sur l'écologie de ces espèces et les compromis impliqués dans le motif des ailes, fournissant ainsi une meilleure compréhension des pressions de sélection qui ont façonné les traits observés chez ces papillons particuliers et les implications possibles en ce qui concerne les variations climatiques, dans le passé et dans l'avenir. Ces résultats remettent en question l'importance cruciale de la couleur pour l'adaptation thermique chez les ectothermes et ouvrent la voie à des recherches plus approfondies sur la thermorégulation métabolique et comportementale, et sur leurs contributions relatives à l'adaptation climatique chez ces espèces.

Résumé en Anglais

In contrast to most butterflies that harbor colorful wings and a wide variety of patterns, some species display transparent wings to various extents, for which light absorption is limited. Transparency raises questions regarding potential costs for vital functions, such as thermoregulation. Moreover, many neotropical clearwing butterflies can be found at high altitudes, with cooler conditions, where species are expected to harbor darker color pattern. Understanding the complex processes involved in thermal adaptation in these butterflies requires an interdisciplinary approach, combining the study of the physical phenomenon of light absorption by the wings, associated with the exploration of thermal needs, tolerance and behavior that contribute to thermoregulation. I focused on the neotropical butterfly tribe Ithomiini (Nymphalidae), which includes circa 400 species, distributed from warm lowlands to colder high elevations in the Andes. First, I undertook a comparative study of the optical and thermal properties of the wings of 42 Ithomiini species, from highly transparent to fully opaque, and from low- to highlands, in a phylogenetic frame. Second, I assessed the capacity for thermal buffering of low and high altitude ithomiine species in their natural environment in the Andes, and their thermal tolerances under extreme temperatures. Third, I reared butterflies from two Panamanian species from the egg stage until adulthood under controlled conditions to quantify the thermally induced plasticity of both wing physical properties and thermal performances of adult butterflies. Our work reveals that while transparent wing patches are less efficient than opaque ones to collect heat from light radiations, the proportion of clearwing species and the proportion of transparent areas increases with altitude, in disagreement with the predictions. Likewise, wing optical properties did not respond to variation in temperature during larval development. The physical properties of the wings are therefore unlikely to contribute to thermal adaptation. By contrast, I found that highland species were more tolerant to cold, and lowland species more tolerant to heat. By taking phylogenetic relationships into account, I show that those thermal tolerances are likely the outcome of both genetic evolution and phenotypic plasticity. This work presents a new interdisciplinary case study of thermal adaptations in a clade of tropical insects and has implications for future studies on the resilience under climate change of such peculiar animals.

Key words :

Ecology, Evolution, Thermal melanism, Thermal adaptation, Altitude, Lepidoptera, Transparency, Plasticity

Résumé en Français

Adaptation thermique des papillons tropicaux aux ailes transparentes

Contrairement à la plupart des papillons qui arborent des ailes colorées et une grande variété de motifs, certaines espèces présentent des ailes transparentes à divers degrés, pour lesquelles l'absorption de la lumière est limitée. La transparence soulève des questions concernant les coûts potentiels pour les fonctions vitales, telles que la thermorégulation. En outre, de nombreux papillons néotropicaux à ailes transparentes se trouvent à haute altitude, dans des climats plus froids, où l'on s'attend à ce que les espèces arborent des couleurs plus sombres. La compréhension des processus complexes impliqués dans l'adaptation thermique de ces papillons nécessite une approche interdisciplinaire, combinant l'étude du phénomène physique de l'absorption de la lumière par les ailes, associée à l'exploration des besoins thermiques, de la tolérance et du comportement qui contribuent à la thermorégulation. Je me suis concentrée sur la tribu néotropical des papillons Ithomiini (Nymphalidae), qui comprend environ 400 espèces, réparties entre les plaines de basse altitude au climat chaud et les hautes altitudes, plus froides, des Andes. Tout d'abord, j'ai entrepris une étude comparative des propriétés optiques et thermiques des ailes de 42 espèces d'Ithomiini, des plus transparentes aux plus opaques, le long du gradient d'élévation, dans un cadre phylogénétique. Deuxièmement, j'ai évalué la capacité des espèces d'ithomines de basse et de haute altitude à ajuster leur température dans leur environnement naturel, ainsi que leur tolérance thermique à des températures extrêmes. Troisièmement, j'ai élevé des papillons de deux espèces panaméennes depuis le stade de l'œuf jusqu'à l'âge adulte dans des conditions contrôlées afin de quantifier la plasticité induite par la température des propriétés physiques des ailes et des performances thermiques des papillons. Nos travaux révèlent que si les parties transparentes des ailes sont moins efficaces que les parties opaques pour collecter la chaleur et les radiations lumineuses, la proportion d'espèces à ailes transparentes et la proportion de zones transparentes sur les ailes augmentent avec l'altitude, en désaccord avec les prédictions. De plus, les propriétés optiques des ailes n'ont pas montré de changement après le développement larvaire à différents traitements de température. Il est donc peu probable que les propriétés physiques des ailes soient impliquées dans l'adaptation thermique chez ces papillons. En revanche, j'ai constaté que les espèces de haute altitude étaient plus tolérantes au froid, tandis que les espèces de basse altitude sont plus tolérantes à la chaleur. En tenant compte des relations phylogénétiques, je montre que ces tolérances thermiques sont probablement le résultat d'évolution génétique et de plasticité phénotypique. Ce travail présente une nouvelle étude de cas interdisciplinaire sur les adaptations thermiques d'un clade d'insectes tropicaux, et a des implications pour les études futures sur la résilience au changement climatique de ces animaux si particuliers.

Mots Clés :

Ecologie, Evolution, Mélanisme thermique, Adaptation thermique, Altitude, Lepidoptera, Transparence, Plasticité