The optical properties, colour and nanostructure of butterfly wings across Neotropical habitats.

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Declaration

The work presented in this thesis was performed at the University of Sheffield and Natural History Museum (NHM) London, between October 2022 and June 2026 under the supervision of Dr. Nicola Nadeau, Dr Blanca Huertas (NHM), Dr Gavin Thomas and Dr Andrew Parnell. I, Sonal Ladwa, confirm that unless stated otherwise, the work is that of my own. I am aware of the University's Guidance on Academic Integrity. No part of this work has previously been presented for an award at this, or any other, university.

Thesis Abstract

Tropical forests contain heterogeneous environments, yet we know relatively little about how this variation shapes insect diversity, distributions and functional traits. Butterflies help investigate these relationships because wing optical properties and nanostructures influence visual signalling, camouflage and thermal exchange. This thesis examines how environmental gradients and habitat structure shape butterfly communities and wing traits, and how these contribute to thermal regulation. Linking community composition with visible, ultraviolet and infrared properties, it explores processes shaping butterfly diversity.

Butterfly abundance and occurrence data across altitudinal gradients and vertical forest strata in Ecuador were used to investigate community composition and turnover. Communities differed among habitats, with species turnover contributing to compositional differences and phylogenetic patterns indicating potential environmental filtering at higher elevations. Multispectral imaging revealed variation in visible, ultraviolet and infrared reflectance associated with habitat use, body size and evolutionary history. Visible reflectance differed between forest strata, with canopy-associated species tending to have darker wings. Relationships between visible and infrared reflectance showed that pigmentation influences properties beyond the visible spectrum. Iridescent species differed in infrared reflectance from non-iridescent species, demonstrating that structural colour modifies thermally relevant optical properties.

Field experiments showed that butterfly thermal state was influenced by air temperature, altitude and habitat exposure, while live thoracic temperatures indicated regulation beyond passive wing heating. Infrared emissivity measurements revealed lower emissivity in iridescent than brown wing scales, suggesting that nanostructural differences influence radiative heat exchange.

These findings demonstrate that butterfly diversity and thermal biology reflect interactions among environmental heterogeneity, evolutionary history and multifunctional wing traits. They highlight the importance of visible and non-visible wavelengths when investigating colouration's ecological and evolutionary functions. Integrating community ecology, phylogenetic analyses, multispectral imaging,

museum specimens and field thermal measurements, this thesis reveals how tropical butterflies interact with heterogeneous environments and how wing optical properties contribute to ectotherm thermal biology.

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Table of contents

Declaration 2

Thesis Abstract 3

Acknowledgements 5

Table Of Contents 7

1.0 Thesis Introduction 9

1.1 Tropical Habitats .....
1.2 Butterfly Diversification Across Tropical Habitats .....
1.3 Colour Signalling And The Environment .....
1.4 Colour, Thermoregulation And Uv .....
1.5 The Optical Properties Of Butterfly Wings .....
1.6 References .....

2.0 Multiscale Community Structure Of Andean Butterflies: Altitudinal And Vertical Stratification Effects 64

2. 1 Abstract .....
2.2 Introduction .....
2.3 Methods .....
2.4 Results .....
2.5 Discussion .....
2.6 References .....
2.7 Supplementary Figures .....

3.0 Multispectral Reflectance In Tropical Butterflies Across Forest Stratification And Altitudinal Range 108

3.1 Abstract .....
3.2 Background .....
3.4 Results .....
3.5 Discussion .....
3.6 References .....
3.7 Supplementary Figures .....
3.8 Supplementary Tables .....

4.0 Altitude And Canopy-Cover Shape Passive Heat Gain And Active Thermoregulation In Tropical Butterflies 153

4.1 Abstract .....
4.2 Introduction .....
4.3 Materials And Methods .....
4.4 Results .....
4.5 Discussion .....
4.6 References .....

4.7 Supplementary Figures .....

5.0 Iridescence Reduces Infrared Emissivity In Butterfly Wing Scales 203

5.1 abstract.....
5.2 introduction .....
5.3 methods.....
5.4 results .....
5.5 discussion .....
5.6 references .....
5.7 supplementary figures .....

6.0 Thesis Discussion.....227

6.1 Environmental effects on butterfly community composition and colour .....
6.2 From wing optical properties to thermal biology .....
6.3 Museum collections as a valuable resource.....
6.4 supplementary figures: .....
6.5 references .....

1.0 Thesis Introduction

1.1 Tropical habitats

Tropical ecosystems support exceptionally high levels of biodiversity across both fauna and flora. Tropical rainforests are particularly diverse, with variation in geography, including altitude, creating a range of environmental conditions and ecological niches (Elton, 1973; Reading et al., 1995; Val et al., 2005; Janzen, 1988). The tropics are located between the Tropics of Cancer (latitude 23.4°N) and Capricorn (latitude 23.4°S) and occupy approximately 40% of the Earth's surface (Val et al., 2005; Feeley & Stroud, 2018), and contain a diverse range of habitats, including tropical savannahs, deserts, monsoon systems and rainforests (Val et al., 2005; Feeley & Stroud, 2018). Although tropical regions are generally characterised by warm temperatures and relatively low seasonality, climatic conditions vary considerably among and within these habitats. This environmental heterogeneity, together with the long-term climatic stability characteristic of many tropical regions, has contributed to the diversification and persistence of highly diverse and specialised lineages (Wolda, 1987; Barlow et al., 2018; Dangles et al., 2009; Burgess et al., 2024).

The tropical Andes of South America, the longest terrestrial mountain chain, are among the most biodiverse regions on Earth (Morellato, 2003). The combination of steep environmental gradients, complex topography and climatic variation provides numerous opportunities for ecological specialisation and diversification. Insects are particularly diverse within Neotropical habitats and play essential roles in the ecosystem, including pollination (Arroyo et al., 1982; Gonzalez & Engel, 2004), driving ecological plant-host interactions (Ehrlich & Raven, 1964; Fine et al., 2013), nutrient cycling and contributions to soil fertility, (Metcalfe et al., 2014; Maldonado et al., 2019), herbivory and a key role in cascading trophic levels (Condon et al., 2008; Greenwood, 1987).

These environmental conditions have also contributed to extensive insect diversification (MacArthur, 1984; Hazzi et al., 2018) through processes including adaptive radiation (Mitter et al., 1988), ecological speciation (Matsubayashi et al., 2010; Rundle & Nosil, 2005), and the evolution of specialised plant- host interactions (Hougen-Eitzman & Rausher, 1994; Mooney et al., 2023; Drès &

Mallet, 2002; Forbes et al., 2017; Ferrer-Paris et al., 2013). Geographical isolation can further promote divergence and speciation among populations across the heterogeneous landscales of the Neotropics (Rull & Carnaval, 2020; Rundle & Nosil, 2005).

The environmental complexity of tropical habitats has also driven the evolution of diverse morphological, physiological and behavioural adaptations. Tropical climates, are variable in to changing climatic conditions such as temperature, rainfall and humidity (Bennett et al., 2023; Duffus et al., 2023; Rull & Carnaval, 2020), and fauna and flora adapted to tropical niches are are highly adapted to these niche tropical environments. Therefore, changes in neotropical niches both directly impact insects through physiological constraints on locally adapted species and indirectly impacts the whole ecosystem through specialised food webs, predation and reproduction, nutrient cycling and more (Bennett et al., 2023; Boyle et al., 2025).

Organisms inhabiting tropical forests must respond to variation in temperature, humidity, solar radiation and habitat structure across relatively small spatial scales. In insects, these pressures can shape traits associated with thermoregulation, camouflage, communication, dispersal and reproduction, including body size, pigmentation, wing morphology and surface structures. Such adaptations can be particularly important in butterflies, whose ectothermic physiology means that environmental conditions directly influence their body temperature and activity. Wing colour and structural properties can simultaneously contribute to visual signalling, crypsis and the regulation of solar energy, demonstrating how morphological traits may have multiple ecological and physiological functions.

Despite their exceptional biodiversity, tropical ecosystems are increasingly threatened by anthropogenic environmental change. Deforestation, agricultural expansion, habitat fragmentation and pesticide exposure have substantially altered Neotropical habitats, reducing and fragmenting the environments on which specialised species depend (Wagner, 2020; Tapia-Armijos et al., 2015; Justino et al., 2026). Climate change presents an additional threat because many tropical organisms are adapted to relatively stable and narrow climatic conditions. Consequently, even relatively small

changes in temperature, precipitation and humidity may alter the environmental conditions to which tropical species are adapted (Bennett et al., 2023; Duffus et al., 2023; Rull & Carnaval, 2020). Species with highly specialised morphological or physiological traits may therefore be particularly vulnerable if environmental conditions shift beyond the range in which these adaptations provide an advantage.

Understanding how tropical species are distributed and how their morphological traits are shaped by environmental conditions is therefore important for understanding the mechanisms that generate and maintain tropical biodiversity. In particular, identifying how environmental gradients such as temperature, solar radiation, humidity and habitat structure influence species distributions and morphological traits can provide insight into the ecological pressures that shape species' adaptations. Understanding the functions of these traits can also help identify how closely species are associated with certain environmental conditions and therefore how they may respond when those conditions change. This is particularly relevant for Neotropical butterflies, which occupy diverse habitats across strong environmental gradients and possess conspicuous variation in wing colour, structure and morphology. Examining these traits alongside environmental variation can therefore provide insight into how species interact with their environments, why particular traits may be favoured under different conditions, and more broadly, indicate how specialised adaptations could influence species' vulnerability to future climate change and habitat loss. Together, these relationships provide a framework for investigating how butterfly communities and wing traits vary across the environmental gradients of Neotropical forests, and how these traits may reflect adaptations to the conditions experienced by species within these habitats.

1.2 Butterfly diversification across tropical habitats

Within the Neotropical region, the Andean countries; Colombia and Ecuador, have a high insect biodiversity, and the world' s highest number of butterfly (*Insecta; Lepidoptera; Pappilionoidea*) species. 43.5% of the planet’s butterfly species are neotropical and are found in extremely biodiverse South American tropical environments. South America, particularly Colombia, Ecuador and the Andean Region, is a well-known hotspot for butterfly diversity (Table 1) (Huertas et al., 2022,

Garwood and Jaramillo, 2022). Butterflies possess a range of qualities that make them a captivating and popular model taxon for evolutionary and ecological research. In particular, their variation in phenotypes, colouration, behaviours, host plant co-evolution, fascinating Mullerian mimicry, and complex phylogeny and evolutionary history have been well studied (Freitas and Brown, 2004, Wahlberg et al., 2009). Making them an important and fascinating group. Over the last few decades, butterfly classification has been developed and refined with a better understanding of phylogeny. Despite previous debate over classification, butterflies are now widely classified into families, subfamilies, tribes, genus, species and subspecies with general consensus (Table 1, Figure 1) (Freitas and Brown, 2004, Wahlberg et al., 2009).

Here, the term 'butterfly' refers to the newer classification of the superfamily Papilionoidea, which places the Hedyloidea and Hesperioidea as sister families under the superfamily Papilionoidea (Heikkilä et al., 2012). The age of the butterfly lineage has been a subject of deliberation for many years, with a general consensus that Papilionoidea diversified in the Tertiary period (around 66mya)(Vane-Wright, 2004). Butterfly fossil records are relatively sparse, and those known are relatively recent, around Oligocene and Eocene (50-15mya) epochs (Nazari et al., 2011). For instance, the oldest fossil confidently described as Lepidopteran is from the early Jurassic period, around 190 million years ago and the oldest known butterfly amber fossil, is dated 52 million years ago (Wahlberg et al., 2013, Vane-Wright, 2004, Wahlberg et al., 2009).

Subsequent studies on the Lepidopteran lineage suggest butterflies must be older than fossil records suggest, as the originally proposed origin date (60-34mya) would imply that angiosperms were already diversified prior to Ditrysia (a suborder consisting of 98% of Lepidoptera), contradicting the notion of co-evolution driving selection on butterflies and their host plants (Regier et al., 2009, Wahlberg et al., 2013). More recent studies looking into the coevolution of plants and their hosts have postulated that butterflies diversified from moths during Cretaceous era (65mya-135mya) in what is known as the 'Age of Flowering plants', where major angiosperm clades originated (Kawahara et al., 2019; Peris & Condamine, 2024; Benton et al., 2022). This older origin date is compatible with the idea that butterflies and plants have been interacting throughout their evolutionary history, which has

driven diversification and formed the long-lived co-evolutionary relationship we see today (Labandeira, 2013; Wahlberg et al., 2013; Borchsenius 1997). It is likely that most butterfly subfamilies originated before the KT Boundary and are older than originally postulated. This newer evidence suggests Nymphalid butterflies diversified over 60mya (94-30mya), and a high proportion of this diversification occurred in neotropical butterflies (Wahlberg 2006; Wahlberg et al., 2013, Nazari et al., 2007).

Table 1: A comparison of butterfly species richness on the planet, richness in neotropical regions and the percentage of the planets' species that are neotropical, per family (*adapted from Garwood and Jaramillo, 2022*).

Family	Species Richness (Planet)	% of worlds butterfly species (Planet)	Species Richness (Neotropical)	% of worlds species (Neotropical)	% of Planets species made up of Neotropical species
Hedylidae	39	0.2	39	0.5	100
Hesperiidae	4113	21.9	2547	31.2	61.9
Papilionidae	603	3.2	140	1.7	23.2
Lycaenidae	5201	27.7	1044	12.8	20.1
Pieridae	1164	6.2	341	4.2	29.3
Riondinidae	1532	8.1	1522	18.6	99.3
Nymphalidae	6152	32.7	2541	31.1	41.3
Total	**18,804**		**8174**		**43.5%**

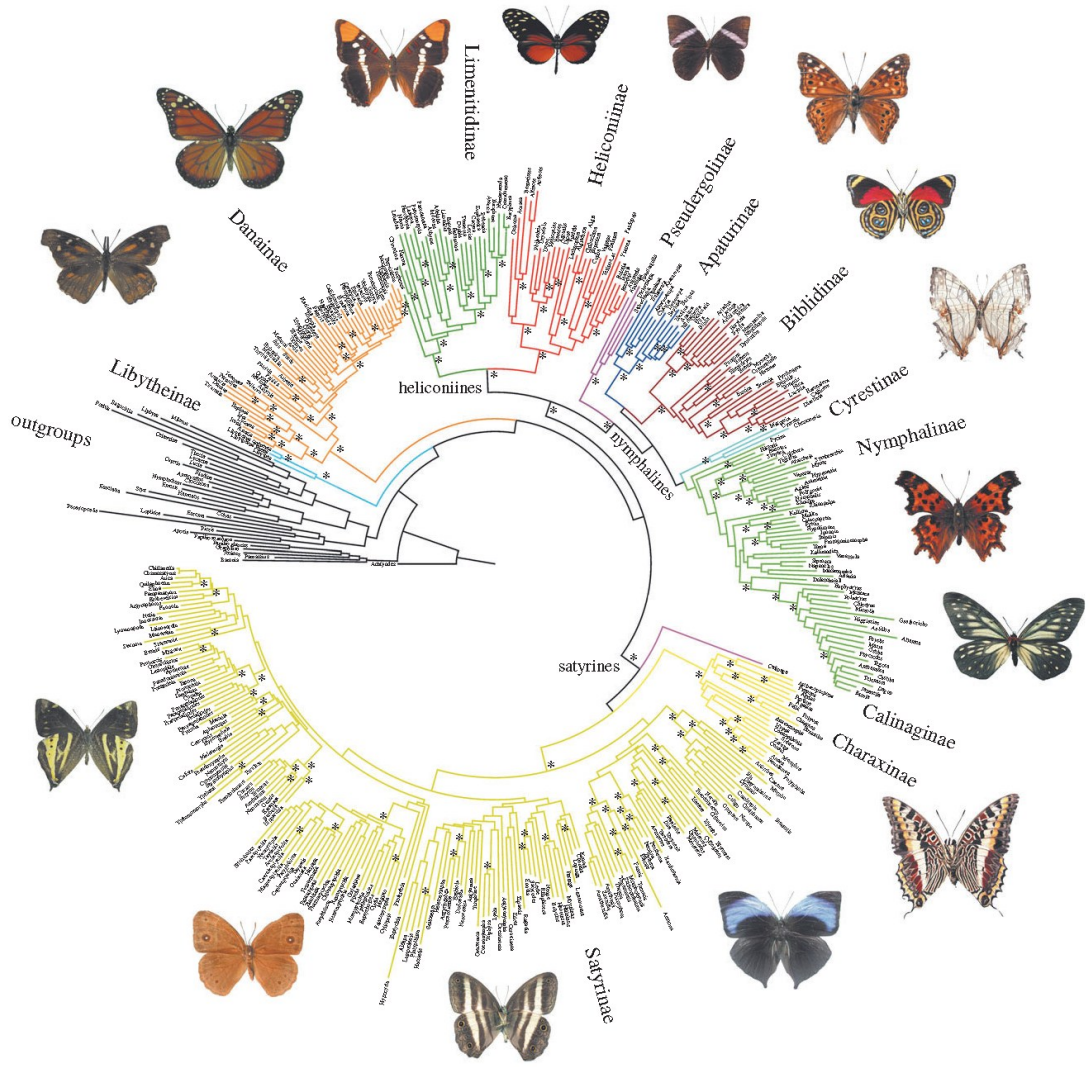


Figure 1: Phylogenetic tree of 400 Nymphalid species spanning 12 sub-families from Wahlberg et al., 2009.

Butterflies have a long-lived co-evolution with host plants for feeding and oviposition (Ackery, 1988; Ferrer-Paris et al., 2013; Wahlberg et al., 2009, Ehrlich and Raven, 1964). For herbivorous insects, host plant choice for both food and oviposition is very important as they must choose accessible plants within their ecological and geographical range (Ehrlich and Raven, 1964, Hodgson, 1993). Interactions between plants and insects have resulted in a co-evolution that is millions of years old (Labandeira, 2013, Crossley et al., 2021). Importantly, plant morphology and availability are an important driver of butterfly diversification, and vice versa (Ehrlich and Raven, 1964; Fine et al.,

2013). Neotropical habitats have a wide and abundant diversity of plant species (Kattan et al., 2004), giving rise to a highly diverse range of plants and hosts and intricate plant-host interactions (Borchsenius 1997; Wahlberg et al., 2009; Ehrlich and Raven, 1964).

The differences in environmental conditions within the Andean region are highly influential on butterfly host plants, through varying temperatures, humidity, light availability, soil composition and more (Wolda, 1987; Morellato, 2003), resulting in cascading effects for both butterfly community, distribution, evolution and morphology (Ackery, 1988). The Andean tropics are an important environment, as it hosts complex and variable geographic habitats, contributing to the wide range of fauna and flora found there (Elton, 1973; Condon et al., 2008; Kattan et al., 2004). The tropics contain habitats spanning variable altitudes, temperatures and humidity, including high altitude cloud forests, humid and dry forests, forest edges, open and closed forest canopies, and arable and grassland areas (Wolda, 1987; Beck et al., 2010; Huertas et al., 2022), all of which create unique and distinct environments where plants and insects have diversified and adapted to these niche and varying conditions.

A key component of the Andean region is the altitudinal gradient which creates distinct changes in temperature, humidity, rainfall and solar irradiation (Wolda, 1987). At higher altitudes, environments become colder, less humid and have a higher level of solar irradiation, compared to the warmer, humid low altitude environments (Laue, 1970; Scheffers et al., 2017; Val et al., 2005; Beck et al., 2008). The difference in topography, vegetation and abiotic conditions across altitudes directly influences butterfly communities and diversity through adaptation, diversification, and host-plant interactions (Montejo-Kovacevich et al., 2020; Rueda-M et al., 2021; Cómbita et al., 2022; Henriques et al., 2022; Pires et al., 2020). Additionally, butterflies have a range of physiological, morphological and behavioural adaptations to survive and thrive in different abiotic gradients, further shaping tropical butterfly diversity, morphology and butterfly communities at different altitudes (Montejo-Kovacevich et al., 2022; Brown, 1982). Certain morphological features such as wing size and shape for flight and manoeuvrability while colour and patterning can affect thermoregulation, camouflage and predator avoidance, potentially influencing species' distributions across environmental gradients.

For example, larger wings may improve flight efficiency and reduce the energetic costs of movement in some environments, whereas smaller wings can increase manoeuvrability in structurally complex habitats (Mena et al., 2020). Darker wing coloration can increase solar energy absorption and may therefore provide a thermal advantage in cooler environments, while lighter or more reflective coloration may reduce heat gain in warmer, more exposed environments (Kingsolver & Watt, 1983; Kang et al., 2021; Ayres and Scriber, 1994). These traits may therefore influence how species respond to differences in temperature, solar exposure and vegetation structure across altitudinal gradients, contributing to patterns of species diversity and community composition.

In tropical forests, temperature and light availability varies with vertical structure. The canopy receives high levels of illumination, whereas the understory and forest floor are characterised by reduced and spatially heterogeneous light conditions due to canopy filtering, creating a darker, dappled light environment (Depauw et al., 2021; Chazdon & Fetcher, 1984; Küppers et al., 1996; Endler.,1992). Whilst forest canopy environments experience higher maximum temperatures, there is much more exposure to winds and weather changes, and variation in temperature here. In contrast, the understory is generally cooler, and more thermally buffered, and has less fluctuation in temperature throughout the day. This variation across stratification not only shapes plant communities but also has a cascading effect on butterfly diversity, distribution, flight, signalling and behaviour and thermoregulation (Schulze et al., 2001; Montejo-Kovacevich et al., 2020; Checa et al., 2014; Mena et al., 2020).

In a study on butterflies, one cannot ignore the vital importance of climate change and the threat to biodiversity it poses. This is paramount for those species evolved to narrow thermal environments and endemic species such as neotropical butterflies. Life has, historically, persisted through a range of global environmental changes and extinctions, but as current climate issues heighten, there are repeated reports of biodiversity and principally invertebrate and butterfly decline (Freitas et al., 2011, Warren et al., 2021, Wagner, 2020, Butchart et al., 2010, Hallmann et al., 2017, Freitas et al., 2014). As conservation and extinction prevention is of rising importance, understanding the evolutionary

mechanics underlying temperature adaptation and thermoregulation will allow us to better predict how neotropical species will cope with future environmental changes.

1.3 Colour Signalling and the Environment

The role of colouration in signalling is complex and has been extensively studied across a wide range of animal taxa, including snakes, frogs, birds, beetles, and butterflies. In butterflies, colouration is subject to multifaceted selective pressures, serving a variety of ecological and evolutionary functions, including signalling. The use of colour as a signal is inherently complex and often encompasses a range of roles in mate signalling during courtship and reproduction, social communication and species recognition, and anti-predatory strategies such as camouflage and warning displays (Cott, 1940; Endler, 1992; Caro et al., 2017; Lloyd & Nadeau, 2021; Brakefield & French, 1999).

Anti-predator signalling may manifest as either conspicuous or cryptic colouration (Medina et al., 2020). In cryptically coloured species, natural selection favours colours and patterns that enhance concealment within the surrounding environment (Guilford, 1988; Smith & Beccaloni, 2008; Endler, 1993). Such concealment often relies on pattern or background matching, which exploits the limitations in predator perception and incorporates a combination of colour, markings, edges, and linear features (Merilaita & Lind, 2005). For example, this strategy is seen in tropical snakes, where selection favours both dorsal and ventral colouration that closely matches environmental backgrounds to reduce detection by predators, or patterning to conceal from predators (Pough, 1976; Stevens & Merilaita, 2011). Similarly, many Lepidoptera employ a form of visual deception through pattern matching, disruptive colouration, and the distortion of wing edges to blend with background colours and textures (Endler, 2006; Kang et al., 2015; Suzuki, 2017; Perveen & Khan, 2024).

In contrast, aposematic colouration involves the evolution of conspicuous signals that advertise unpalatability or toxicity to potential predators (Caro & Ruxton, 2019; Loeffer-Henry et al., 2025; Fiedler & Brehm, 2021). Warning signals are often complemented by behavioural displays. For instance, some snakes enhance their apparent threat by flattening their heads or producing warning sounds, such as tail rattling. Coral snakes exemplify aposematic signalling through their distinctive

banded patterns, typically consisting of red, yellow, and black coloration (Buasso et al., 2006; Banci et al., 2020). Here, such overt colouration has successfully evolved to deter predators, yet the expression of conspicuous signals may vary with body size, as some aposematic juvenile snakes exhibit a weaker signal than larger adults (Niskanen & Mappes, 2005). Poison frogs provide another well-documented example, where vivid colouration functions as a clear signal of unpalatability in tropical animals (Vences et al., 2003; Sztatecsny et al., 2012). In tropical moths, Tiger and Lichen moths exhibit a higher frequency of warning patterning at lower altitudes and where predation pressure is greater, highlighting the role of environmental factors such as altitude in shaping signalling strategies (Fiedler & Brehm, 2021). Aposematic signalling is also widespread in tropical butterflies, where features such as eyespots act as deterrents to predators (Pinheiro et al., 2016; Lyytinen, 2001; Lürig et al., 2025; Wee & Monteiro, 2017; Stevens, 2005). Additionally, flash colouration in butterflies where angle-dependent or bright signals are revealed during flight or wing movement, can confuse predators in a manner similar to aposematic displays (Kim et al., 2020; Drinkwater et al., 2022). Aposematic signalling relies on positive frequency-dependent selection, where the effectiveness of a warning signal across multiple species increases with its prevalence in individuals within a population (Sherratt, 2008; Pfennig et al., 2001). The evolution of these aposematic signalling systems is frequently accompanied by the emergence of mimicry. In snakes, Batesian mimicry occurs when non-venomous species resemble venomous counterparts, thereby deceiving predators (Sena & Ruane, 2022; Pfennig et al., 2001; Kikuchi et al., 2014). Müllerian mimicry, by contrast, involves multiple unpalatable species converging on a shared warning signal, as observed in frogs, bumblebees, wasps, and other taxa (Symula et al., 2001; Sherratt, 2008; Williams, 2007). Tropical butterflies provide a particularly well-studied example of Müllerian mimicry, forming complex mimicry rings in which colouration signals unpalatability and reinforces predator learning (Pérochon et al., 2025; Pinheiro et al., 2016; Mallet & Gilbert Jr, 1995; Enciso-Romero et al., 2017; Pinheiro, 2003).

Beyond anti-predatory functions, colouration also plays a critical role in reproductive signalling. Bright and saturated pigmentation often reflects individual health and can influence reproductive

success. In birds, carotenoid-based colouration is condition-dependent, as carotenoids are required for antioxidant and immune functions (Blount, 2004). Individuals in better condition can allocate any excess carotenoids to ornamental traits, thereby signalling health status during mate selection (Lindsay et al., 2011). Additionally, melanin-based traits such as bill colouration may indicate hormonal and immune conditions, as melanin production is linked to testosterone levels (Karubian et al., 2011). Avian mate signalling is therefore highly dynamic, involving interactions between multiple ornaments during courtship (Lindsay et al., 2011; Hill, 2000; Blount, 2004; Karubian et al., 2011). Similarly, amphibian colouration is influenced by both physiological condition and environmental factors where the chromatophore activity and diet affect signal expression (Hettyey et al., 2009; Brenes-Soto et al., 2017). In tropical tree frogs, colouration may play a more significant role than body size in mate choice, as smaller males can sometimes exhibit brighter signals than larger males (Hettyey et al., 2009). Dietary carotenoids can offset the costs associated with smaller size by enhancing colour saturation and signalling nutritional condition (Hill, 2011; Brenes-Soto et al., 2017; Sztatecsny et al., 2012; Hettyey et al., 2009). Butterflies also rely on colour for mate recognition, and visual mating cues utilising ultraviolet, iridescent, and polarised light signals (Morehouse & Rutowski., 2010; VanKuren et al., 2025; Papke et al., 2007; Sweeney et al., 2003; White et al., 2015). Importantly, the functions of colouration are rarely independent; instead, they are often traded-off or multifunctional, simultaneously contributing to anti-predation, camouflage, and reproductive signalling (Finkbeiner et al., 2014; Rojas et al., 2018).

The selection of colouration, particularly in the context of signalling, is closely linked to environmental factors such as light availability and thermal conditions (Macedo et al., 2020; Moyen et al., 2006; Goldenberg et al., 2022). For example, a study of sexually dimorphic colouration in beetles found that diurnal species exhibit brighter colouration than nocturnal species, with males being significantly brighter than females in diurnal taxa (Ribeiro et al., 2024). This pattern suggests that visual signals are shaped by the light environment, as their effectiveness diminishes under low-light conditions (Ribeiro et al., 2024). More broadly, the light environment influences both the evolution of visual systems and the selection of effective colour signals (Warrant & Johnsen, 2013). Both anti-

predatory mechanisms (e.g., camouflage) and intraspecific communication (e.g., mating signals) are affected by the light gradients across vertical forest stratification. For instance, Morpho butterflies utilise striking blue iridescence as a polarised signal for conspecific communication; however, the conspicuousness of this signal depends strongly on environmental context and viewing angle (Kemp, 2007; Sosa Espinosa, 2025). Consequently, the same iridescent signal may be conspicuous to intended receivers under some viewing conditions while remaining relatively inconspicuous from other angles, potentially creating a trade-off between effective signalling and crypsis. The context dependence of iridescence as a signal, illustrates how a single visual trait can have different ecological consequences depending on how it is viewed and perceived. Such trade-offs are evident in other taxa. In dung beetles, iridescent colouration can function both in camouflage and as a signal of mate quality, while also playing a role in male–male competition (Vulinec, 1997). Importantly, these visual functions may also interact with the physical properties of iridescent structures. Variation in iridescent colouration has been associated with environmental temperature, suggesting that structural colour may have consequences beyond visual communication and could potentially influence thermal balance and, consequently, species distributions across altitudinal gradients (Davis et al., 2008).

1.4 Colour, Thermoregulation and UV

Animal colouration has also been found to have a range of secondary thermoregulatory effects alongside primary signalling functions, adding complexity to the selection on animal colouration (Stuart-Fox et al., 2017; Kingsolver, 1987). Chameleons are a classic example, changing colour for social signalling and thermoregulation through dispersing or aggregating melanin within their cells (Stuart-Fox & Moussalli, 2008; Smith et al., 2016). The speed and extent of these colour changes reflect the intensity of selection for responses to shifts in environmental conditions, particularly thermal conditions. In birds, the relationship between pigmentary and structural colours in feathers, alongside their physiological demands of thermoregulation, results in diverse optical properties where colour contributes to both communication and thermal regulation. For example, the precise arrangement of melanin in feathers can affect a bird's visible appearance, which is important in signalling, but also the structural heat absorption properties of feathers (e.g., darker tips on wings for

efficient flight in cold climates) (McGraw et al., 2003; Rogalla et al., 2022; Prum, 2004). This secondary thermal effect of colouration adds to the complexity of selection on and function of colouration. Therefore, for butterflies complexities arise with the function of colour for visual colour properties such as signalling and crypsis when there may be additional thermoregulatory properties caused by certain colours (Mallet & Singer, 1987; Kingsolver, 1987; Stavenga & Arikawa, 2006; Silberglied, 1984).

Butterflies produce some of the most striking and diverse colours in nature and have variable wing colouration across lineages and populations. Insects are under selection for traits that enable them to cope with varying abiotic conditions (Ayres and Scriber, 1994, Davis et al., 2005; Rosa & Saastamoinen, 2020). Butterflies, who are ectothermic and require higher body temperatures to fly, have evolved various thermoregulatory mechanisms (Bladon et al., 2020). These include pre-flight contractions of thoracic muscles, radiative cooling and absorption, detection of solar radiation, hemolymph flow around 'living' wings, and differential colouration on dorsal and ventral sides of wings (Kingsolver, 1987, Tsai et al., 2020). In animals that are adapted to live in abiotically constrained areas, biogeographical environments are important in shaping traits, that often impact colouration and visual signal effectiveness such as body size (Bergmann's Rule), appendage size, (Allen's rule) and wing pigmentation (Verma & Arya, 2023; Holliday, 2002; McQueen et al., 2022; Nudds & Oswald, 2007; Tilkens et al., 2007; Moen, 1968; Schelar, 1966; Ellers & Boggs, 2004).

Two ecological rules regarding colouration across biogeographical gradients are Gloger's Rule and Bogert's rule/Thermal Melanism Hypothesis (Kettlewell, 1973). Gloger's rule suggests that individuals from warmer, more humid environments are more likely to be darker in colouration (Delhey, 2019; Goldenberg et al 2022). Gloger's rule has been observed predominantly in birds and mammals. where functions of the rule are explained by increased pigmentation (and more intense colours generally) being selected for as adaptations to warmer but low light environments, protection from UV exposure or parasites. Whilst this was initially a hypothesis about endotherms, Gloger's rule has since been expanded to ectotherms, who rely on external temperature to heat their bodies, and thus have evolved different strategies to regulate body temperature across variable environments

(Goldenberg et al 2022). Bogert's rule or, the thermal melanism theory, suggests the opposite, that darker colouration in animals is more likely to occur in those adapted to colder regions (True, 2003; Clusella-Trullas & Nielsen, 2020; Cooney et al., 2022; Storniolo et al., 2025; Bittner et al., 2002). The contrasting predictions of these two rules reflect two different proposed selective mechanisms. Gloger's Rule predicts increased melanisation towards warmer and more humid environments, whereas the Thermal Melanism Hypothesis predicts increased melanisation towards colder environments because darker surfaces absorb solar radiation more efficiently. This latter mechanism may be particularly important for ectotherms, such as butterflies, because increased absorption of solar energy can increase body temperature and potentially facilitate activity in cooler environments. In butterflies, the degree of melanisation in butterflies has been correlated with environmental temperatures (Guppy, 1986; Watt, 1969; Trullas et al., 2007; Riley, 1997). Darker body colouration (melanism) is functional in juvenile stages, as darker individuals have increased absorption of thermal/heat energy, which can accelerate caterpillar development in cooler temperatures. Melanic adults may express increased active heat absorption, facilitating wing circulation and flight; beneficial when individuals adapted to high elevations are exposed to lower temperature environments or allowing them to occupy larger biogeographical ranges (Ayres and Scriber, 1994, Davis et al., 2005; Nylin & Gotthard, 1998). Alternatively, increased melanin results in more efficient cooling down, due to increased rates of heat emission, and thus can be beneficial to individuals at higher body temperatures (Kettlewell, 1973, True, 2003; Ossola et al., 2023).

Beyond visible signalling, butterflies, like other insects and animals, utilise ultraviolet light in signalling (Stella & Kleisner, 2022; Brunton & Majerus, 1995). Melanin absorbs and dissipates harmful ultraviolet radiation, giving improved protection against UV damage (Blumthaler et al., 1997; Brenner & Hearing, 2008; Goulson, 2009; Rosa & Saastamoinen, 2020; Stella & Kleisner, 2022). In high-exposure environments, such as forest canopies or high altitude, or across wider altitudinal ranges, selection may favour increased melanisation to limit UV damage (Blumthaler et al., 1997; Goulson, 2009; Stella & Kleisner, 2022; Kingsolver & Koehl, 1985). On the flipside, lighter

colouration may be selected for at higher temperatures where risk of overheating is more likely (Ellers and Boggs, 2003). While melanin provides increased protection from harmful radiation through absorption of UV, it can also diminish the use of visible colour in signalling or create opportunities for signalling via UV signals and contrast patterning. Because ultraviolet also plays a key role in signalling, (Stella & Kleisner, 2022; Riley, 1997) increased UV reflectance may be favoured for communication, indicating a potential trade-off between UV protection and signalling. Increased melanisation also enhances infrared absorption, amplifying heat gain. This occurs via internal conversion, whereby melanin absorbs UV and converts it into heat (Brenner & Hearing, 2008; Nakashima & Yoshihara, 1989; Ruyter & Øysæd, 1982). Although the thermoregulatory consequences of this process remain poorly studied in insects, it likely imposes an additional trade-off, whereby selection for UV protection (i.e., increased melanin) reduces visible signalling capacity, impacts the efficacy of pattern contrast signalling and increases heat load, linking pigmentation, thermoregulation, and signalling in a complex evolutionary balance.

Wing darkness is comprehensively expected to change as an adaptation to environmental altitudes and temperatures, and in Lepidoptera darker colours are more generally associated with colder environments. This is seen in adult Monarch butterflies Lepidoptera:Nymphalidae *Danaus plexippus,* Swallowtail, Lepidoptera:Papilionidae *Parnassius phoebus* and Clouded Yellows Lepidoptera:Pieridae *Colias* (Guppy, 1986, Stelbrink et al., 2019, Davis et al., 2005, Ellers and Boggs, 2003, Watt, 1968). Melanism is widely variable between species, as dark melanic patches can enhance other colour or pattern signals, whilst also exhibiting secondary thermoregulatory constraints on the individual. To date, much of the literature focuses on specific subspecies or genera, consequently, little comparative work has been published on overall trends of colouration and trends in melanism across butterfly groups and how this correlates with altitude and abiotic ecological factors (True, 2003; Kang et al., 2021). Furthermore, whilst the assumption that melanin increases absorbs more heat, the thermoregulatory consequences of colouration selected for signalling is often neglected in research.

Many colouration studies focus on the visible spectra, and secondary optical effects of infrared wavelengths (beyond 700nm) are not often accounted for (Stuart-Fox et al., 2017). For butterflies particularly, this is an important aspect due to their sophisticated thermoregulation of the wings separately from the body (Tsai et al., 2020). Wavelengths beyond the visible spectrum and into the infrared spectrum are an important aspect to consider, as butterflies are exposed to solar radiation, where over 50% of the solar radiation that reaches the earth is infrared (Iqbal, 2012; Vignola et al., 2019). The shifts in temperature across altitudes in tropical habitats are well documented, alongside the knowledge that even small shifts in altitude have large differences in solar irradiation (Montejo-Kovacevich et al., 2020; Janzen, 1967; Scheffers et al., 2017). This in turn may have consequences for butterfly optical properties across tropical altitude gradients and forest vertical stratification. Thermal adaptation in tropical butterflies is often studied in relation to metabolic processes to maintain body temperatures, however much less research has been conducted on the thermal consequences of wing morphology and the effects of wing colour on thermoregulation (Kingsolver & Watt, 1983; Ashe-Jepson et al., 2023; Dongmo et al., 2021). Aside from active processes, butterfly wings have unique and complex material properties that are likely to affect the temperature and thermal absorption of wings.

1.5 The Optical Properties of Butterfly Wings

The material optical properties of wings are important in further understanding the role of colour in the thermoregulation of wings. The various optical properties of wings and their capability to manipulate ultraviolet and infrared wavelengths is due to pigmentation and the presence of intricate biological colour-producing nanostructures (Kinoshita et al., 2008). Optical material properties, such as that in butterfly wing nanostructures, have been utilised in the development of bioinspired materials. These optical mechanisms have inspired various industrial applications such as in the design and fabrication of materials for screen display technology, industrial carbon fibre networks, thermal and medical sensors and energy harvesting and storing applications such as photocatalysts and solar cells and panels, contributing to bio-inspired renewable energy, biofuels and medical health

monitoring (Bosi et al., 2008; Rodríguez et al., 2018; Chung et al., 2012; Osotsi et al., 2021; Wang et al., 2026; Zhang et al., 2014).

For years, the function and production of colour in animals have been well researched. Colours in nature are generally the result of one of two physical phenomena: pigmentation or structural colouration (Kinoshita et al., 2008). In pigmented materials, the molecular composition causes differential absorption of visible wavelengths. The colour produced depends on the distinct wavelengths that are absorbed, and unabsorbed wavelengths are those that are reflected and transmitted to the eye. For example, black objects absorb light of all wavelengths equally, incomplete absorption of all wavelengths gives rise to grey colouration, and reflection of all wavelengths produces white (Fox, 1953; Tilley, 2020).

Pigmentary colour is produced when light energy interacts with a surface, the molecules are in a state of resonance: a vibratory motion that is particular to each compound. Light excites electrons within the molecule to a different level, and the resonating molecule can absorb light with a frequency complimentary to the frequency of resonance, producing colour (Fox, 1953; Kinoshita et al., 2008; Duncan, 1940; Tilley, 2020). For instance, a compound with a short resonance can absorb blue and violet colours and reflect longer wavelengths such as yellow. Red compounds have a longer resonance value and can absorb wavelengths in the blue and green range, reflecting red. Colours are not always reflected and absorbed within spectral ranges, for example purples reflect combinations of blue and red whilst absorbing the rest (Fox, 1953; McGraw et al., 2005; Tilley, 2020). With pigmentary colour, the interaction of light with a particle involves an exchange of electrons, which creates colours seen in materials such as metals, pigments, and dyes (McGraw et al., 2005).

Alternatively, colour can be produced structurally, whereby light interacts with structures in a material and, unlike the above mechanism, does not involve the exchange of elections (Kinoshita et al., 2008). In nature, many colours are structural, where there are physical interactions between light and nanostructures within the material that result in the production of colour.

Early recognition of structural colour was observed in peacock feathers by Robert Hooke in 1665 (Hall, 1990). In 1704, Issac Newton published 'Opticks' in which he discussed the mechanics behind light, colouration in nature and white light (Newton's Rings) (Newton, 1952). In 1873, the formation of the Electromagnetic Theory gave rise to experiments by Hertz (in 1884), on electromagnetic waves, deepening understanding on the properties of light, reflection, refraction, interference and diffraction (Kinoshita et al., 2008; D'Agostino, 2000; Ball, 2007). In 1895, there was a divide in hypotheses structure-colour (interference) vs surface-colour (pigment), and it was postulated that colour in animals is produced by dyes (Caygill., 2020; Kinoshita et al., 2008). However, Lord Rayleigh proposed that colour comes from structure and between 1924-25, research on colour-producing structure in animals supported interference theory (Kinoshita et al., 2008). The invention of the electron microscope in 1931 elevated the study of structural colour and was utilised in studies on blue colouration in ivory-breasted pitta feathers (1939), blue iridescence in Morpho butterflies, iridescence of hummingbird feathers, scarabaeid and jewel beetles (Freundlich., 1963; Anderson & Richards., 1942; Greenewalt et al., 1960; Neville and Caveney., 1969; Seago et al., 2009). In 1991, Ghiradella characterised a complete nanostructure of wing scales, enabling further understanding of structure and colour in butterflies (Ghiradella., 1991).

Optical nanostructures in animals vary in their refractive index, causing shifts in optical paths, ultimately resulting in light scattering, reflecting and refracting uniquely across different wavelengths (Prum et al., 2006; Fox, 1953; Ingram and Parker, 2008). There are a range of optical phenomena that give rise to structural colour, such as photonic crystals, multilayer interference, thin-film interference, and diffraction gratings, however in animals, colouration is often enhanced by combinations of these (Prum et al., 2006; Kinoshita et al., 2008; Vukusic & Stavenga, 2009; Tilley, 2020). In some instances, structural and pigmentary colour are utilised simultaneously. Pigments, such as melanin packaged in melanosomes; responsible for black, brown and grey colours and carotenoids for reds, pinks, oranges and yellows, are embedded in the chitinous nanostructures, which tunes the colouration produced (Riley, 1997; McGraw et al., 2005; Ghiradella, 1991; Vukusic & Stavenga, 2009; Tilley, 2020).

Butterfly wings consist of uniform, periodic structures composed of structured chitin and air. Scales are generally arranged in alternating rows, occurring in two forms: cover scales that overlap the ground scales (Ghiradella, 1991; Vukusic et al., 2004; Parnell et al., 2018). Scale nanostructures are characterised by rows of raised longitudinal ridges, connected at intervals via cross-ribs. These are supported by pillars of columnar trabeculae functioning as spacers between the upper and lower laminae (Figure 2). Pigmented scales have pigment molecules located within the structure, either in granules or within the trabeculae (Ghiradella, 1991, Parnell et al., 2018). Despite this general structure, different scales have a unique nanostructure that differentially interacts with light to produce colour, such as iridescence (Parnell et al., 2018).

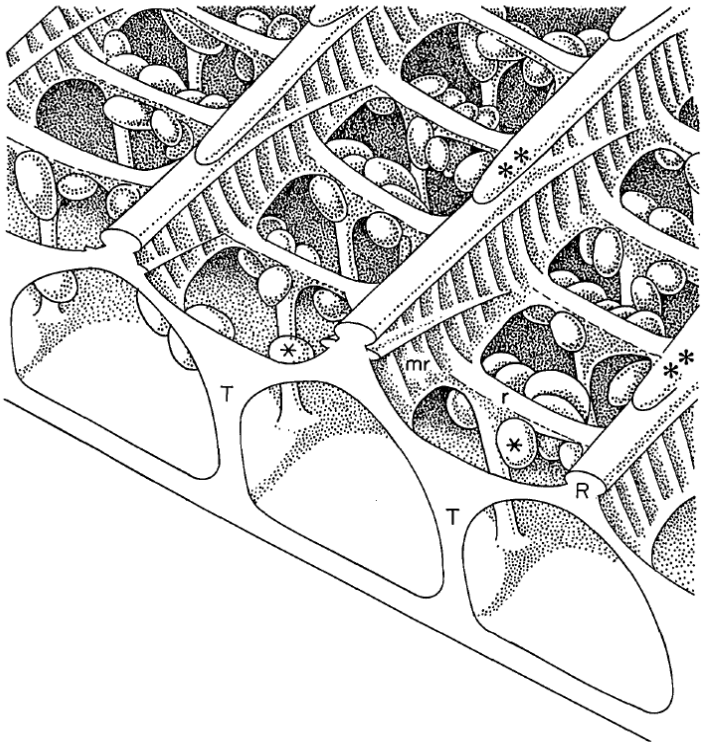


Figure 2: A detailed diagram showing the nanostructure of a butterfly wing scale from 'Light and colour on the wing: structural colours in butterflies and moths' (Ghiradella, 1991). This diagram shows the trabeculae (T), ridges (R), lamellae (**), ribs (*r*), microribs (*mr*). The pigment granules (*) are shown but are not present in all scales.

For instance, white is a structural colour, where expansive optical scattering of light waves is facilitated by unique nanostructures (Burg and Parnell, 2018; Zhu et al., 2009). Alongside iridescent and white structures, black is both melanistic and evidenced to be structurally assisted. Black

colouration is known to absorb all wavelengths and convert this into heat energy, but due to unique nanostructuring, can vary in levels of absorption and reflection. This suggests the ability for these structurally assisted black structures to have selective absorption of longer wavelengths, as an adaptation to different environments or temperatures (Vukusic et al., 2004).

Iridescence is the result of a form of coherent scattering: thin film-interference (Tchenka et al., 2025; Prum et al., 2006; Parnell et al., 2018; Zhu et al., 2009). Iridescence can be described as "a change in hue or brilliance with angle of observation and illumination", as in a soap bubble. Simplistically, thin film interference is where incident light hits a film with a specific thickness and is partially reflected and refracted off the top and bottom layers of a film (Burg and Parnell, 2018). The partially reflected/refracted light, scatter in different optical paths, with the same direction, giving rise to interference between the scattered rays. The interference of scattered light is caused by the phase difference between the reflected rays and is dependent on the optical path distance (Figure 3). Constructive interference can occur when two rays of the same wavelength are reflected from the top layer of a thin film and a bottom layer of the thin film (Miaoulis & Heilman, 1998). Two optical paths could experience a phase length difference when passing through the thin film, where one ray is delayed and thus out of phase with the other. This results in destructive interference of light (Miaoulis & Heilman, 1998). The reflected colour of iridescent materials is highly dependent on the optical mechanism of the nanostructures, the thickness of the film, its refractive index, and the angle of incidence: all of which results in this striking optical iridescence that changes depending on the viewing angle (Figure 4). (Burg and Parnell, 2018; Ghiradella, 1991; Prum et al., 2006).

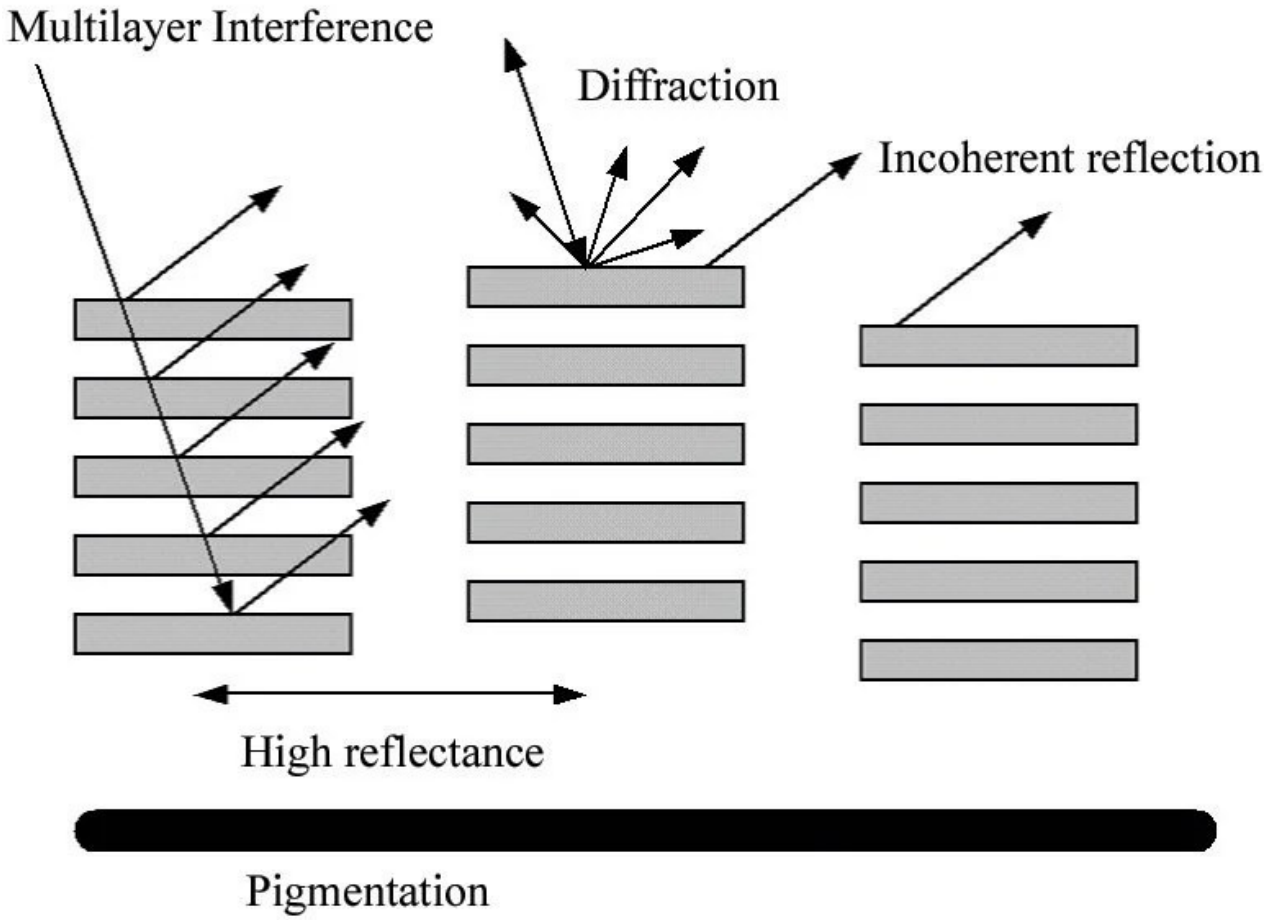


Figure 3: Principal contributions to iridescence function in “the separate lamellae model” from ‘Rendering Methods for Models With Complicated Micro Structures’ (Iwasawa et al 2004)

Many studies looking at film thickness and optical properties focus solely on visible wavelengths (~ 400 nm - 700 nm). Whilst some studies do include shorter infrared wavelengths (~800 nm - 900 nm max wavelength), the interference of infrared wavelengths is often overlooked in these studies (Teolis et al., 2007), and so relatively little is known about the thin-film inference in the infrared spectrum.

Thermoregulation in butterflies is not limited to solar absorption, but is also facilitated through emission of heat in the mid- infrared (MIR) wavelengths (Kirchhoff's Law of Thermal emission; Wien’s Law of energy distribution) (Robitaille, 2009; Biermann et al., 2002: Krishna et al., 2020). The optical properties of butterfly wing structures have been observed up to 1.1µm which encapsulates ~83% of the solar spectrum. Wing structures have been observed to scatter MIR separately from NIR, UV and VIS wavelengths. Furthermore, a comparison between 4 species found

a difference in the reflectivity of structures between all four, suggesting specific nanostructure changes may be responsible for variation in reflectivity (Munro et al., 2019; Krishna et al., 2020). Alongside this, thermal systems tend to favour photonic structures with broadband reflectance to allow for infrared emittance, as this form of surface reflects light in the longer infrared wavelengths, however it is still possible for narrowband photonic structures to influence emissivity of infrared wavelengths.

There is a level of contention in the literature regarding the role of iridescence in solar absorption/reflectance, whereby some suggest solar absorption is enhanced by nanostructures, and others suggest the opposite (Bosi et al., 2008). Observations on solar energy reflectance spectra absorbed by chitin layers, found the thickness of the film determines the proportion of solar radiation that is absorbed in Lepidoptera:Papilionidae, *Papilio palinurus*. This implies iridescent structures are 'solar collectors' as these structures had increased solar absorption. Other studies found iridescent structures enhanced solar absorption in different areas of Lepidoptera:Nymphalidae *Archaeoprepona meander* wings (Heilman & Miaoulis, 1994; Berthier, 2005). Conversely, studies on three Lepidopteran species concluded the presence of iridescent structures decreased, not increased, the amount of light absorbed (i.e., increased reflectance). Further studies also found that photonic-crystal structures reduced absorption of light in blue regions of butterfly wings (Koon & Crawford, 2000, Biró et al., 2003). Notably, most of these studies used small sample sizes and did not utilise full solar spectrum data, and thus no consensus on the role of iridescent structure in solar absorption has been reached. Studies on larger sample sizes observed higher solar absorbance in iridescent species compared to non-iridescent species, and similar NIR reflectance in both (Bosi et al., 2008). Furthermore, little research has been done on the role of melanin within butterfly wing nanostructures, and how pigmentation deposition impacts infrared absorption and reflection from the wing. As butterfly colouration occurs as a result of both pigmentary and structural processes. the scale nanostructures, often embedded with melanin or pigments, impact the optical properties of the wing as melanin exhibits a high level of light absorption, and across a broad wavelength range. However, the explicit effects of melanin in butterfly nanostructures, on the absorption of infrared is unclear.

Overall Thesis Aims and Hypotheses:

The overarching aim of this thesis was to investigate how tropical environmental variation is associated with butterfly community composition and wing optical properties, and to explore the potential ecological and thermoregulatory functions of variation in wing colour and structure. Specifically, I asked whether butterfly communities differ across altitudinal and vertical habitat gradients, whether wing optical properties vary predictably with the environments occupied by species, and whether differences in wing colour and structure have consequences for thermal regulation. I further aimed to determine whether these patterns were consistent across phylogenetically related species and whether variation in infrared properties could provide an additional mechanism through which wing structure influences thermal exchange. Together, these questions address how butterfly communities and wing traits are associated with environmental variation across both broad altitudinal habitats and fine-scale vertical forest microhabitats.

The first data chapter investigated whether tropical Nymphalid communities assemblage and diversity differ in composition and diversity across altitude and forest strata. I used data from a trapping project across four sites in the Ecuadorian rainforest, which provided a dataset of butterfly community composition and habitat associations for 148 species. I tested whether communities would be more distinct across altitudinal gradients than between the canopy and understory, reflecting the greater environmental differences expected across altitude. I examined alpha and beta diversity of communities and the relative contributions of species turnover and nestedness to differences among communities. Phylogenetic diversity was also examined to determine whether environmental gradients were associated with non-random patterns in the evolutionary composition of communities. Finally, species-specific associations with altitude and forest stratum were quantified to provide habitat distributions that were used in subsequent chapters. This chapter established the ecological and phylogenetic context in which differences in butterfly wing traits could be investigated.

The second chapter used these species-specific habitat associations to test whether butterfly wing optical properties vary with the environments occupied by species. Multispectral imaging of butterfly specimens at the Natural History Museum, London were used to quantify visible, ultraviolet and

infrared reflectance across 148 species, including both iridescent and non-iridescent butterflies. I tested whether wing reflectance differed between species associated with different altitudes and between canopy and understory associated species, and whether these relationships were influenced by wing size and iridescence. As wing colour can have multiple functions, including communication, camouflage and thermoregulation, this chapter aimed to determine whether broad-scale environmental variation was associated with consistent differences in butterfly wing optical properties. Phylogenetic comparative analyses were used to account for shared evolutionary history and determine whether observed patterns persisted after accounting for phylogenetic relatedness.

The third chapter investigated whether differences in butterfly colour and wing properties have consequences for thermoregulation. Through fieldwork, I obtained measurements of air and wing temperatures at mid and low altitudes and differing canopy cover. Using two Heliconius species, I compared dead wing and live body temperatures across low and mid altitude environments and under different levels of canopy cover. I asked whether butterfly body and wing temperatures differed from ambient temperature, whether thermal responses varied with altitude and solar exposure, and whether patterns observed in dead wings were reflected in live butterflies. By comparing live individuals with dead wings, this chapter aimed to distinguish thermal effects associated with passive wing material properties from those arising through active behavioural or physiological thermoregulation.

The fourth chapter investigated whether differences in wing scale structure are associated with infrared emissivity. Here, we complemented the multispectral data obtained from digital photography, with spectroscopy measurements to quantify the emissivity of wing patches across a smaller subset of species. Using spectroscopy across a subset of species, I compared the emissivity of black, iridescent and underlying membrane surfaces across visible and infrared wavelengths. I asked whether iridescent (structural coloured) and melanic (pigmentary) scales differ in their capacity to emit visible and infrared wavelengths and whether these differences vary at different wavelengths. This chapter examined a potential thermal function of wing scale structure that is not captured by visible colour alone, providing further insight into how structural differences among butterfly wings may influence the exchange of thermal radiation.

Together, the four data chapters investigate butterfly diversity and wing optical properties across complementary spatial and functional scales. The first chapter establishes how butterfly communities and species' habitat associations vary across environmental gradients; the second examines whether these habitat associations correspond to differences in wing optical properties; the third tests the potential consequences of wing properties for thermoregulation in living butterflies; and the fourth examines infrared emissivity as a potential mechanism underlying differences in thermal radiation exchange. Collectively, these approaches aim to improve understanding of how environmental conditions, evolutionary history and wing optical traits interact to shape the ecology and thermal biology of tropical butterflies.

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2.0 Multiscale Community Structure of Andean Butterflies: Altitudinal and Vertical Stratification Effects

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conducted all analysis that is presented here and wrote of the manuscript, supervised by N.N. S.L. and N.J.N. edited and finalised the manuscript.

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2. 1 Abstract

Understanding how shifts in the environment structure tropical biodiversity is important for determining why species occur where they do and how communities are assembled across variable landscapes. We examined how Neotropical butterfly community assemblages differ across altitudes and forest stratification, as these have varying environmental conditions including temperature, humidity, light exposure and weather. Using rarefied richness, alpha diversity, β-diversity, and phylogenetic diversity (PD), we tested whether communities differed between Andean slopes, across altitudes, and across vertical stratification within the forest. Diversity was higher in lowland sites compared to mid-altitude on both sides of the Andes, consistent with increased environmental filtering. β-diversity indicated that many species were unique to individual sites, supporting strong environmental structuring. Understory communities generally showed higher species richness than canopy communities, despite the canopy's greater light availability, suggesting harsher or more variable canopy conditions limit species occupancy. Species overlap was greater between strata within

a single site than between sites. PD patterns mirrored richness: lowlands supported more distantly related lineages, whereas higher altitudes and most canopy habitats contained more closely related species. Overall, butterfly assemblages were distinct across both altitude and vertical stratification, likely shaped by a combination of geographic isolation, environmental filtering, and microscale habitat conditions. These findings demonstrate how both broad scale environmental gradients and fine-scale forest structure contribute to patterns of butterfly diversity and species–habitat associations across the Andes, providing an important baseline for understanding how biodiversity is partitioned across tropical mountain forests.

2.2 Introduction

Tropical butterflies have a unique biodiversity and possess a range of qualities that make them a captivating and popular model taxon for evolutionary and ecological research. In particular, they are model organisms for the study of biodiversity, variation in phenotype, invertebrate behaviour, host plant co-evolution, Mullerian mimicry. Their complex phylogeny and evolutionary history have been well studied (Freitas and Brown, 2004, Wahlberg et al., 2009). Over the last few decades, butterfly classification has been developed and refined with a better understanding of phylogeny (Freitas & Brown, 2004; Wahlberg et al., 2009). Nymphalidae (brush-footed butterflies) are the most diverse family of butterflies: currently consisting of 8162 species worldwide and making up 32% of the Earth's butterflies (Table 1) (Garwood & Jaramillo, 2021). Nymphalid butterflies are found in all terrestrial regions of the world, except Antarctica. Adults have striking colouration and a well-documented phylogeny (Ackery, 1988; Freitas & Brown, 2004; Wahlberg et al., 2009). 43.4% of the planet's Nymphalid species are neotropical and are found in extremely biodiverse South American tropical environments (Huertas et al, 2022; Garwood & Jaramillo., 2021). South America, particularly Colombia, Ecuador and the Andean Region, is a well-known hotspot for butterfly diversity (Morellato, 2003; Huertas et al., 2022; Garwood & Jaramillo., 2021). The Andes of South America, the longest chain of terrestrial mountains, is one of the most biodiverse areas (per square meter) on the planet. Andean countries Colombia and Ecuador are among the first-ranked with the highest number of butterfly species. The Andean tropics hosts complex and variable geographic habitats, contributing

to the wide range of fauna and flora found there (Morellato, 2003; Kattan et al., 2004). The tropics contain habitats spanning variable altitudes, temperatures and humidity, including high altitude cloud forests, humid and dry forests, forest edges, open and closed forest canopies, and arable and grassland areas (Huertas et al., 2022), all of which vary significantly in temperature, rainfall, humidity and vegetation. The variety of habitats across the Andean region are important drivers of butterfly community distribution (MacArthur, 1984; Hazzi et al., 2018; Bonebrake et al., 2010).

Butterflies are one of many insect groups that have a long-lived co-evolution with host plants for feeding and oviposition (Wahlberg et al., 2009; Ehrlich & Raven, 1964). For herbivorous insects, host plant choice is a key determinant of both feeding and oviposition success, as individuals are constrained to select plants that are accessible within their ecological and geographical range (Ackery, 1988; Hodgson, 1993). Complex interactions between plants and insects have resulted in a co-evolution that is millions of years old (Hougen-Eitzman & Rausher, 1994; Agosta, 2006; Labandeira, 2013; Mooney et al., 2023). Plant morphology and availability are major drivers of butterfly diversification, and butterflies in turn influence plant evolution (Ehrlich & Raven, 1964). As light availability, soil quality, humidity, and temperature shift across altitudinal gradients and vertical stratification in tropical forests, host plant communities vary accordingly, with direct consequences for butterfly distributions, given that host plant availability is a key driver of butterfly community composition (Ackery, 1988; Scheffers et al., 2017).

Differences in abiotic conditions across altitudinal gradients are likely to influence butterfly diversification (Beirão et al., 2021). Lower altitudes are typically warmer and more humid, whereas higher altitudes are cooler and often experience increased precipitation and stronger winds. In addition to shaping host plant distributions, these abiotic factors directly influence the butterfly assemblages that can persist within distinct altitudinal niches. Higher altitudes generally support lower vegetation diversity and present harsher climatic conditions, including lower temperatures and higher rainfall. Butterfly abundance is therefore expected to decrease at higher altitudes (Wolda, 1987). Consequently, niche occupation at higher altitudes is likely restricted to species adapted to these climatic and vegetative constraints (Beirão et al., 2021). In contrast, lower altitudes, characterized by

warmer temperatures and greater plant diversity, provide more permissive environments that support higher species richness and allow a broader range of butterfly lineages to diversify (MacArthur, 1984).

At a microhabitat scale, vertical stratification within forests encompasses a range of environmental conditions, further driving variation in butterfly assemblages at finer spatial scales (Ribeiro et al., 2012; Elton, 1973; Schulze et al., 2001). The forest understory is buffered against wind and experiences relatively stable microclimatic conditions, resulting in a narrower thermal range and reduced light availability due to shading by overlying canopy layers. As a result, the understory effectively functions as a thermal and light insulator within the forest environment (De Frenne et al., 2019). In contrast, the forest canopy is brighter and more exposed, receiving higher levels of solar radiation and experiencing greater thermal variability due to reduced buffering from weather conditions. The canopy also supports greater plant species richness, driven by increased light availability and a wider diversity of light, water, and nutrient conditions (Bohlman, 2015; Basham et al., 2023; Scheffers et al., 2017). Predators of butterflies, including birds and bats, are generally more abundant in the canopy, whereas amphibians and small mammals tend to occur at lower abundances at these heights (Basham et al., 2023).

Despite being more thermally variable and exposed, the canopy's increased host plant availability and higher light levels (conditions that may enhance visual signalling) have been associated with higher butterfly diversity relative to the understory (Fordyce & DeVries, 2016). Large-scale studies in Amazonian tropical forests similarly report greater Lepidoptera abundance in upper forest strata compared to the understory (de Souza Amorim et al., 2022), and rarefied species richness has also been found to be higher in the canopy (DeVries, 1988). However, patterns across vertical strata are not consistent across systems, as in Bornean rainforests, for example, fruit-feeding nymphalid abundance was lower in canopy habitats. This difference likely reflects a variation in fruit availability or the difference in fruit trees and their positioning in the forest strata between Bornean and Amazonian rainforests (Schulze et al., 2001). These contrasting findings highlight the need to empirically test patterns of community diversity, abundance, and richness across vertical strata, rather

than assuming consistent canopy–understory differences (Mena et al 2026). As abiotic and biotic drivers of community structure at microhabitat scales vary across altitudinal gradients and Neotropical niches, vertical stratification should therefore be considered alongside altitudinal differences in community composition (Fordyce & DeVries, 2016; Bennett et al., 2015). Furthermore, it is likely that the differences in communities across stratification are dependent on altitude, as canopy and understory environments are further impacted by the general environmental conditions across altitudes. For example, at higher altitudes where temperature is lower and weather is more extreme, exposed canopy environments may be more challenging than canopy environments at lower, warmer altitudes.

Butterfly communities are often driven by species turnover, where species are replaced by new ones rather than smaller communities being a subset of more species rich ones (Ki et al., 2025; dos Santos et al., 2017). This is expected for communities at different altitudes, where there is environmental filtering and niche specialization between spatially and environmentally different communities. Less is known about species turnover between understory and canopy communities. Within each altitudinal site, nestedness may occur with the separation of communities between understory and canopy where subsets of the altitudinal communities' form across varying vertical stratification. Alternatively, as environments across forest vertical stratification have notable differences in temperature, weather exposure, humidity and plant-life, it is plausible that communities across the vertical gradient also undergo environmental filtering or niche specialisation across these microclimates (Mena et al 2026; Fordyce & DeVries, 2016; Bennett et al., 2015). Further understanding of how communities are partitioned is important as this reveal whether mechanisms of species' turnover or community nestedness play a role, not only across altitude but also across the smaller vertical scale within forests. This provides greater insight into how environmental heterogeneity at different spatial scales shapes community structure and can provide a more comprehensive understanding of community structure whilst highlighting the importance of considering vertical stratification alongside altitude.

More broadly, butterfly assemblages are shaped by a range of interacting environmental and biotic factors (Bennett et al., 2015; Bohlman, 2015; Bonebrake et al., 2010). Community structure thus

provides an important indicator of how environmental conditions shape butterfly distributions. There are cascading effects of light availability, humidity, temperature, and rainfall, that influence host plant availability (Ackery, 1988; MacArthur, 1984), which in turn affect butterfly distributions. These abiotic conditions also determine the efficacy of morphological adaptations (Wenda et al., 2021), including metabolic thermoregulation (Heinrich, 1993), flight morphology (Mena et al., 2020), and reproductive strategies (Blau, 1980). Finally, behavioural adaptations and signalling strategies, such as shade seeking and conspecific signalling, further mediate species distributions across geographical and environmental gradients (Bonebrake et al., 2010; Wenda et al., 2021). Butterfly distributions are therefore shaped by a complex suite of environmental, physiological, and behavioural factors, underscoring the importance of examining community structure across both altitudinal gradients and vertical forest strata.

This study aims to determine how distinct communities are between larger altitudinal gradients but also between forest strata. Due to the presence of the environmental barrier of the Andean mountain range, we hypothesise that communities on the Eastern and the Western Andes will be the most distinct. It is expected that communities, in both the East and West, will be significantly distinct between altitudes as environmental conditions and plant availability distinctly changes with altitude. As butterflies can move more freely between forest understory and canopy, but there remains a strong difference in environmental conditions across vertical stratification, we expect communities to be less distinct, but still unique between vertical strata. Here, we characterise the alpha and beta diversity of communities across altitudes and between the understory and the canopy. We assess the phylogenetic diversity to understand how conserved different linages are across altitude and vertical communities. Further, we partition beta diversity into turnover and nestedness components to determine whether differences in community composition are primarily driven by species replacement or by differences in species richness.

We further examine phylogenetic diversity to determine whether patterns of species richness are also reflected in the evolutionary relatedness of communities. Finally, we characterise the altitudinal distributions and vertical stratification preferences of 148 species of neotropical Nymphalidae.

Together, these analyses examine how broad-scale altitudinal gradients and fine-scale vertical habitat structure contribute to patterns of butterfly community composition and species distributions across Neotropical forests.

2.3 Methods

To obtain accurate habitat data for a set of neotropic nymphalid species, existing data from four trapping sites in Ecuador were used. This dataset includes a total of 168 species. The trapping site and community distribution metrics in this chapter are repeated in subsequent chapters.

Trapping Sites and Data

Data on butterfly communities was obtained from monitoring programmes carried out across four sites in Ecuador at mid (900m and 1500m) and low (250m and 400m) altitudes, on the east and west sides of the Andes (Table 1, Figure 1). The sites are as follows: Yasuni National Park, a humid tropical rainforest in Pastaza and Orellana provinces (250m, 00430S, 80093W), Wildsumaco Lodge Sumaco, a humid cloud rainforest in Napo province (1500m, -.674638889 S, -77.60886111 W, 1500m), Mashpi Rainforest Reserve, a cloud forest in Pichincha province (900m, 0957 N, 785237 W) and El Durango, mountainous and heavily forested in Durango province (400m 10053 N, 783619 W).

Sampling was conducted across an altitudinal range of 250–1500 m, with one study site representing each elevation. These sites were selected as they encapsulate a reasonable altitudinal and sampling was conducted under the same trapping protocols. This design allowed us to examine butterfly communities across the different altitudes, while recognising that altitude was represented by a single location. The study therefore provides an assessment of how butterfly assemblage and diversity varied across sites, rather than a prediction of butterfly diversity at higher altitudes beyond the sampled range. Consequently, differences among elevations may also reflect site-specific environmental characteristics, and the effects of altitude cannot be separated from site effects

Trapping data was provided from a study which ran from 2014 to 2017. A total of 32 Van Someren Rydon bait traps were paired in the understory (0.37– 0.92 m) and the canopy (11–27.3m), and each

pair were spaced between 47–127m from one another (Checa et al., 2022) (Fig. 1). Traps were baited with either fermented banana or rotted shrimp to increase the range of fruit and carrion feeding butterflies (Whitworth et al., 2018; Checa et al., 2019). Traps were also paired such that an equal number of shrimp and banana traps were always included in each trapping effort. As we were primarily interested in the distribution and habitat preference of each species, we did not include bait type in our analysis. Although bait type may influence species distributions, the data did not contain sufficient replication or balanced sampling across the different bait types to accurately assess this effect. In particular, the number of samples and repeated observations for individual bait types varied among sites and stratification, making it difficult to disentangle the effects of bait type from differences in sampling effort and habitat. Including bait type could have introduced bias and produced unreliable estimates of species' habitat associations. Singletons (species represented by only one individual per site) were removed for all subsequent analysis, as they create unreliable estimates of trapping frequency. A single capture provides limited information on the relative abundance or trapping frequency of a species within a site. Including these records could disproportionately influence the estimates of community composition and similarity. These records may represent rare species or incidental captures and provide limited evidence of consistent representation within the sampled community. Removing singletons reduced the potential for these rare, potentially incidental captures to misrepresent patterns of community structure and diversity.

Table 1: The four sites from which ecological monitoring data was used for this thesis.

Site	**Side of the Andes**	**Altitude**	**Altitude category**
Mashpi	West	900m	Mid
Durango	West	400m	Low
Wildsumaco	East	1500m	Mid
Yasuni	East	250m	Low

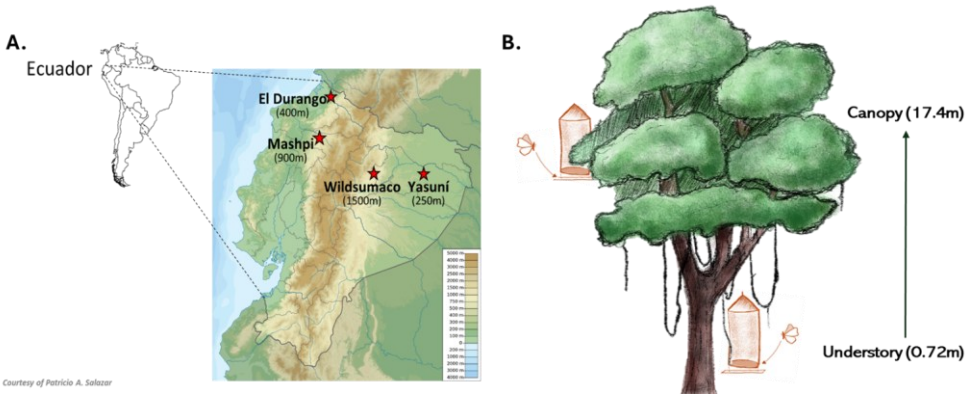


Figure 1: A: The four trapping sites in Ecuador: Yasuni National Park, 250m; Wildsumaco Lodge, at 1500m; Mashpi Rainforest Reserve, 900m and El Durango, 400m, courtesy Patricio A. Salazar. B: An illustration of the trapping positions for each trap pair in the understory and the canopy, and the median distance from the ground (in meters).

Community Composition: Alpha (α) Diversity

Species richness was rarefied in the vegan package (Oksanen et al., 2025), to account for unequal sampling effort through measuring the number of species with a standardized sample size. A rarefaction curve was generated and plotted using the 'rarecurve' function in the vegan package. We tested the rarefied species richness at a given sample size to analyse richness regardless of sample size. We rarefied species richness community matrices for species trapped across both altitudes and at the two levels of vertical stratification, which were used in the following analysis to minimise biases due to unequal sample size.

Alpha diversity using both the Shannon-Weiner index and the Simpson index were calculated on the rarefied data to describe the local diversity of each community between sites. These methods use a measure of community heterogeneity. The Shannon-Weiner (H) index (Shannon, 1948) indicates the disorder or uncertainty of each species within a community whilst considering the count, abundance and evenness, where higher H value indicates higher uncertainty, suggesting increased diversity. The Simpson (D) index (Simpson, 1949) uses the number of species alongside the relative abundances of species to account for the similarity between two sites or communities and is a measurement of the

probability of randomly selected species from a pool. Simpson's index ranges from 0 to 1 where the higher D indicates a lower diversity. Simpson's diversity index (D), places greater weight on dominant species and likely emphasises community dominance structure rather than species richness. Although both indices are presented for completeness, we focus our interpretation on the Shannon index, which better captures changes in overall community diversity across sites. Hill numbers (Hill, 1973; Chao et al., 2014) were calculated from the rarified data as an alternative representation of α-diversity measures as the effective number of species (Equation 1). Hill numbers provide a framework for expressing the diversity of communities while accounting for both species' richness and relative abundance, with different values of q determining the weighting given to common and rare species. Here q0 represents the total number of species, q1 represents the exponential of Shannon diversity which weights species proportional to their abundances, and q2 represents the inverse Simpson index (Equation 1), which focusses on highly abundant species and discounts rarer species. Thus, the q1 and q2 Hill numbers correspond to transformations of the Shannon and Simpson diversity measures, respectively, rather than representing additional independent diversity indices.

q=0 $^{0}D = S$

q=1 $^{1}D = \exp\left(-\sum_{i=1}^{S} p_i \ln p_i\right) = \exp(H')$

q=2 $^{2}D = \frac{1}{\sum_{i=1}^{S} p_i^2}$

Equation 1: Hill number equations where S is the number of species, p_i is the relative abundance of the ith species and q is the order of diversity. When q=0, ^{0}D corresponds to species richness. When q=1, ^{1}D corresponds to the exponential of Shannon entropy and represents the effective number of species based on the observed relative abundances. When q=2, ^{2}D corresponds to the inverse Simpson concentration, with greater weight given to the most abundant species (Hill, 1973; Chao et al., 2014).

Phylogenetic Diversity of Communities

A phylogeny from Charzot et al., 2021, was used. The initial tree included 2,880 Nymphalid species (~45% of current diversity) (Chazot et al., 2021). Using the 'ape' R package (Paradis & Schliep.,

2019), the phylogeny was trimmed to include the species in this dataset. To fill the gaps of missing phylogenetic data, 18 species were replaced with data from the existing phylogeny, by substituting another species within the same genus (as they only have one species missing within the genus). Finally, 20 species with no available phylogenetic data were removed as they could not be placed, resulting in a pruned, rooted phylogeny containing 148 species.

Faith's phylogenetic diversity (PD; Faith, 1992) was calculated for each community using the pd function in the picante package (Kembel et al., 2010). This was done for communities at each site, and vertical stratification at each site. Because raw PD is inherently dependent on species richness, we quantified standardized effect sizes of PD (SES PD) using the ses.pd function, which compares observed PD to null communities of identical species richness generated by randomizing species identities across the phylogeny. Null distributions were generated from 999 randomizations using the "taxa.labels" null model. SES PD values therefore reflect departures from random phylogenetic structure after accounting for differences in species richness among communities.

Phylogenetic clustering was measured using the Net Relatedness Index (NRI) and Nearest Taxon Index (NTI) metrics, based on the mean phylogenetic distances (MPD) using the ses.mpd function in the picante package (Kembel et al., 2010). The mean phylogenetic distances were calculated with the independent-swap null model, which randomised the community matrix and enabled us to test whether the phylogenetic clustering is stronger than expected by random chance.

Community Composition: Beta Diversity

The beta diversity was used to quantify the composition of taxa between communities. Sørensen dissimilarity provides a measure of compositional dissimilarity between two assemblages based on shared and unique species and therefore assumes there is comparable sampling effort between sites. The dissimilarity values were calculated twice; 1. Between all pairs of sites at low and mid altitudes on the east and west side of the Andes and 2. between pairs of communities at understory and canopy at each site.

To further partition beta diversity into turnover and nestedness components we used the betapart package (Baselga et al., 2025), which can decompose Sørensen dissimilarities for abundance data. However, the "beta.pair.abund" function requires species with non-zero abundances shared across sites. Because our data contained few shared species with non-zero abundance across all sites, abundance-based partitioning could not be computed. Instead, we calculated pairwise presence-absence nestedness and turnover using the "beta.pair" function, for each site and stratum. This function computes Sørensen dissimilarity between all pairs of assemblages and partitions the total beta diversity into two additive components: (i) spatial turnover, representing species replacement between assemblages, and (ii) nestedness-resultant dissimilarity, representing differences in richness where species-poor assemblages form subsets of richer ones. Calculations were performed separately for each site and vertical stratum, allowing us to assess whether differences in community composition across altitudes and forest strata were primarily driven by species replacement or by nested loss or gain of species. This resulted in a breakdown of the turnover and nestedness components of beta diversity.

Species distribution: Altitude and Vertical Stratification

To quantify species distributions across low and mid altitudes, relative abundance within each site was quantified. Because sampling effort varied across sites (2014–2017), species counts were standardised as relative abundances per site (species count divided by total individuals captured). Relative abundances were used to quantify altitudinal distributions using a scaled index, a/(a+b), where *a* and *b* represent relative abundance at low and mid altitude, respectively. For species occurring on both sides of the Andes, this was calculated separately for eastern and western sites. Vertical stratification (understory vs. canopy) was characterised using a hierarchical Bayesian model in BayesPref (v1.0) (Gompert & Fordyce, 2012). Because individuals can move more freely between vertical strata than between altitudes and so tended to be caught in both canopy and understory traps, this approach allowed a more accurate estimation of preference from paired trap data. BayesPref models estimate the occurrences from canopy and understory traps as a binomial distribution (1 = canopy preference, 0 = understory). Parameter estimation used 4,000 MCMC generations with a

1,000-generation burn-in and default priors (Uniform 1–50 population multiplier; Dirichlet population-level preference parameters (concentration vector = default)). Models were looped for each species using matrices of trap-specific counts. Posterior preference estimates were extracted and averaged across trap pairs for each species.

2.4 Results

Community Composition: Rarefaction

Rarefaction analysis indicated differences in species richness among sites that are not attributable to sampling effort. Most sites approached an asymptote (flattening of the curve) shortly after the minimum shared sampling effort (black line in Fig. 2), for both site and vertical stratification comparisons. Yasuni (250 m) appeared to be slightly under-sampled, as it exhibited the highest species richness and required the greatest sampling effort before the curve began to level off, suggesting that some diversity may remain undocumented. Overall, rarefaction curves were smooth, did not overlap among sites, and showed clear indications of approaching a plateau, indicating adequate sampling coverage across sites (Fig. 2). Rarefied species richness allowed for the comparison of species numbers despite uneven sample sizes (Fig. 3). Richness decreased from low to mid altitudes on both the east and west sides of the Andes (Fig 3a). Across vertical stratification, differences between understory and canopy richness varied with altitude: in the western low community (400 m), canopy was more species-rich than the understory, whereas the opposite pattern was observed at the other sites (Fig. 3b).

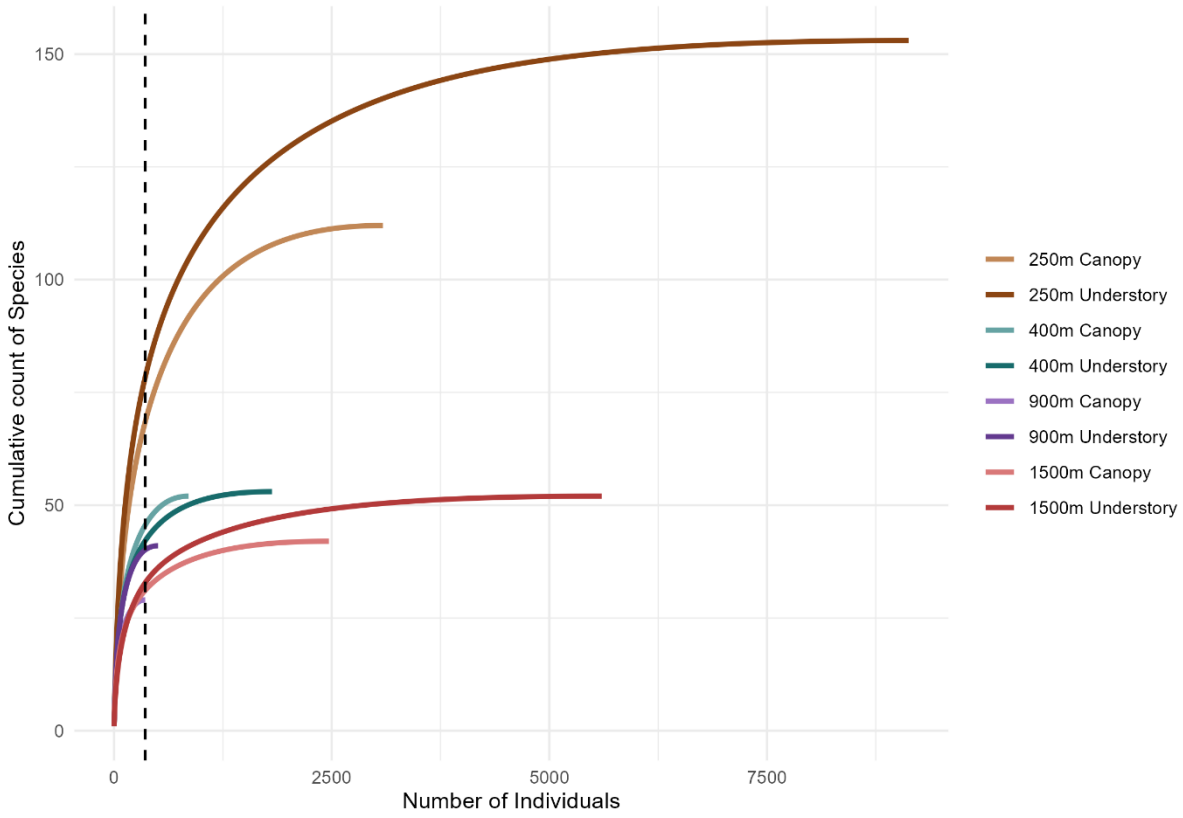


Figure 2: Rarefraction curve for species across altitude and vertical stratification at each site, where the x axis shows the number of sampled individuals and the y axis shows the cumulative number of observed species. The curves are coloured by site. The dotted line represents the observed (raw) number of species.

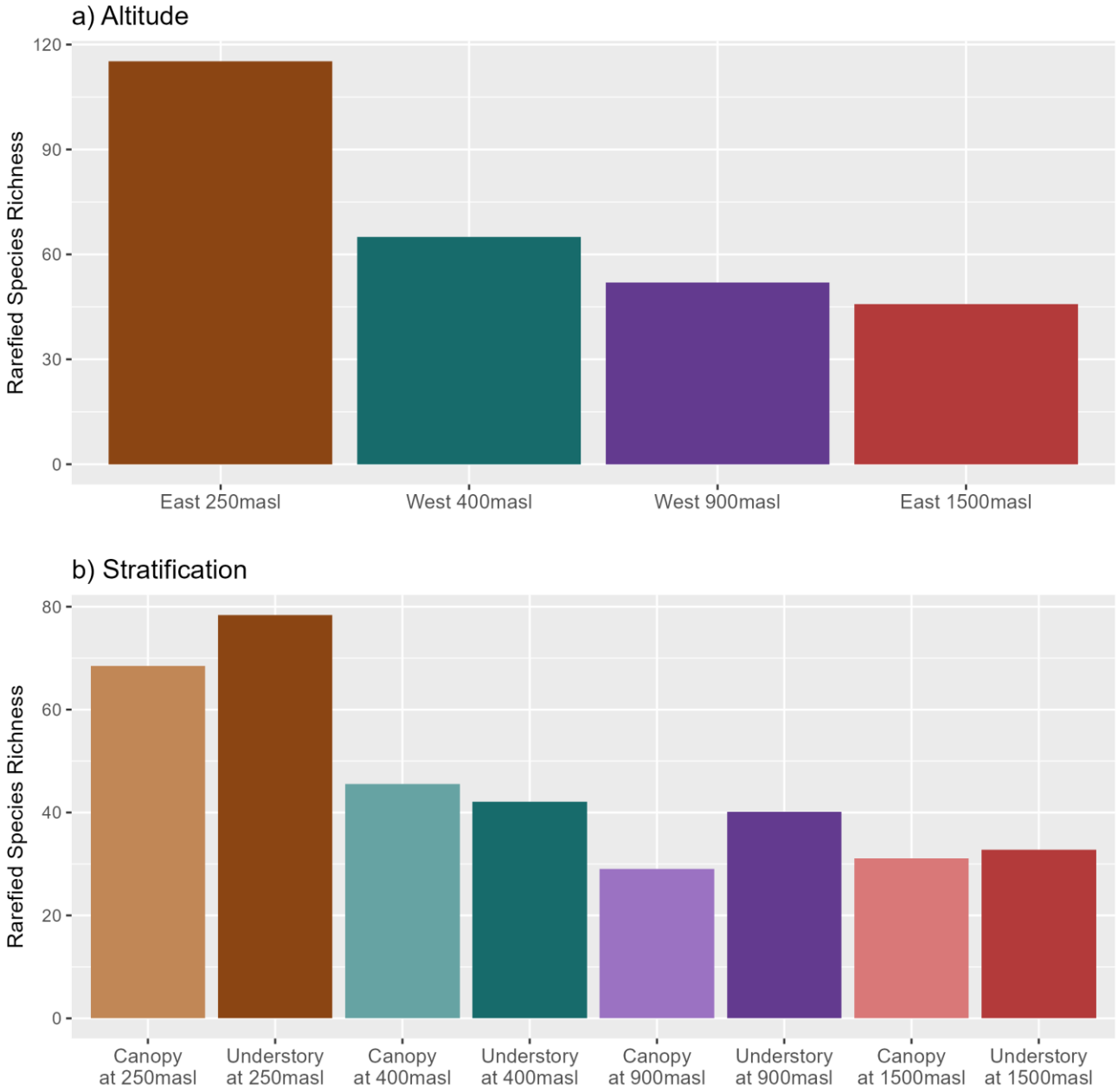


Figure 3: The rarefied species richness across communities at different a) altitudes and sides of the Andes and b) at different altitudes and vertical stratification. Green bars indicate the western Andes communities, and orange the eastern Andes.

Community Composition: α-Diversity

The Shannon–Wiener index (H) was used to estimate α-diversity across sites and revealed a clear pattern, with Yasuni (250 m) exhibiting the highest diversity and Wildsumaco (1500 m) the lowest

(Supplementary Table 1; Fig. 4a/4b). As Shannon diversity is sensitive to both species richness and evenness, this pattern indicates greater overall community heterogeneity at lower altitudes. Mashpi (900 m), despite having the smallest sample size (number of species = 74), exhibited substantially higher Shannon diversity than Wildsumaco (Supplementary Table 1). Simpson's diversity index (D) showed the same trends (Supplementary Table 1). To further assess α-diversity patterns, Hill diversity indices were calculated across diversity orders (q = 1 and q = 2) (Supplementary Table 1; Fig. 4b). These indices revealed a consistent decline in diversity with increasing altitude, corroborating the patterns observed using the Shannon index. Yasuni (250 m) consistently exhibited the highest α-diversity, while Wildsumaco (1500 m) exhibited the lowest, supporting the robustness of the Shannon-based results (Figure 4a/4b).

The same α-diversity metrics were subsequently applied to communities stratified by altitude and vertical position (Supplementary Table 2; Figure 4c). Hill diversity indices here, also showed consistent trends across Shannon and Simpson diversity, reinforcing the reliability of Shannon diversity estimates across strata (Figure 4c/4d). The understory at Yasuni (250 m) exhibited the highest diversity overall, whereas the understory at Wildsumaco (1500 m) exhibited the lowest. Within sites, vertical patterns varied: at the western lowland site, diversity was very similar between understory and canopy communities. At the eastern mid altitude site, canopy communities were more diverse than the understory, whereas the opposite pattern was observed in western mid altitude and eastern lowland sites. Notably, patterns in Shannon diversity (H) and Hill–Shannon diversity (q = 1) (Figure 4) closely mirrored those observed for rarefied species richness (Figure 3), indicating that differences in diversity across sites were primarily driven by changes in species richness rather than shifts in relative abundance or dominance structure.

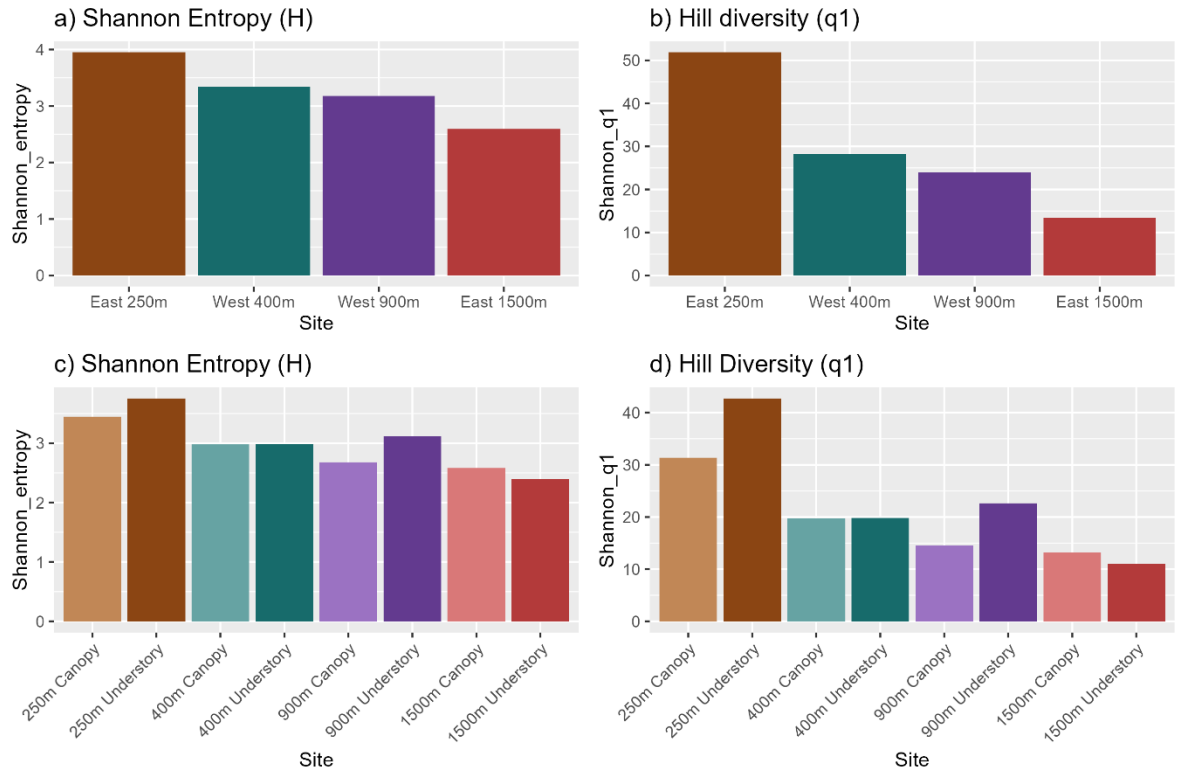


Figure 4: The Shannon Entropy and Hill q1 for each community. Green bars indicate the western Andes communities, and orange the eastern Andes.

Phylogenetic Diversity and Clustering

Phylogenetic diversity (Faith's PD) across sites and strata, showed largely the same patterns as species richness and other measures of diversity (Figure 5a). To correct for the expected relationship between PD and species richness we calculated standardized effect sizes of Faith's PD (SES). Although standardized effect sizes of phylogenetic diversity were calculated to control for differences in species richness, residual correlations with richness remained, likely reflecting strong phylogenetic structuring across the altitudinal gradient and the small number of assemblages. Such residual dependence is expected and does not invalidate SES-based comparisons. For all sites, SES metrics were between -1.99 and 1.99 and therefore within the distribution of expected phylogenetic diversity given the observed species richness (Figure 5b). SES PD remained correlated with species richness across sites, where inspection of null distributions indicated that this pattern was primarily driven by the strong phylogenetic clustering observed at mid altitude, rather than by differences in species

richness. When partitioning by stratification, only the canopy community at Mashpi (900masl, West) showed significant clustering of related species, whilst the other communities all were within the bounds of expectation (Figure 6). Overall, these results suggested limited variation in phylogenetic structure across altitudes and vertical strata.

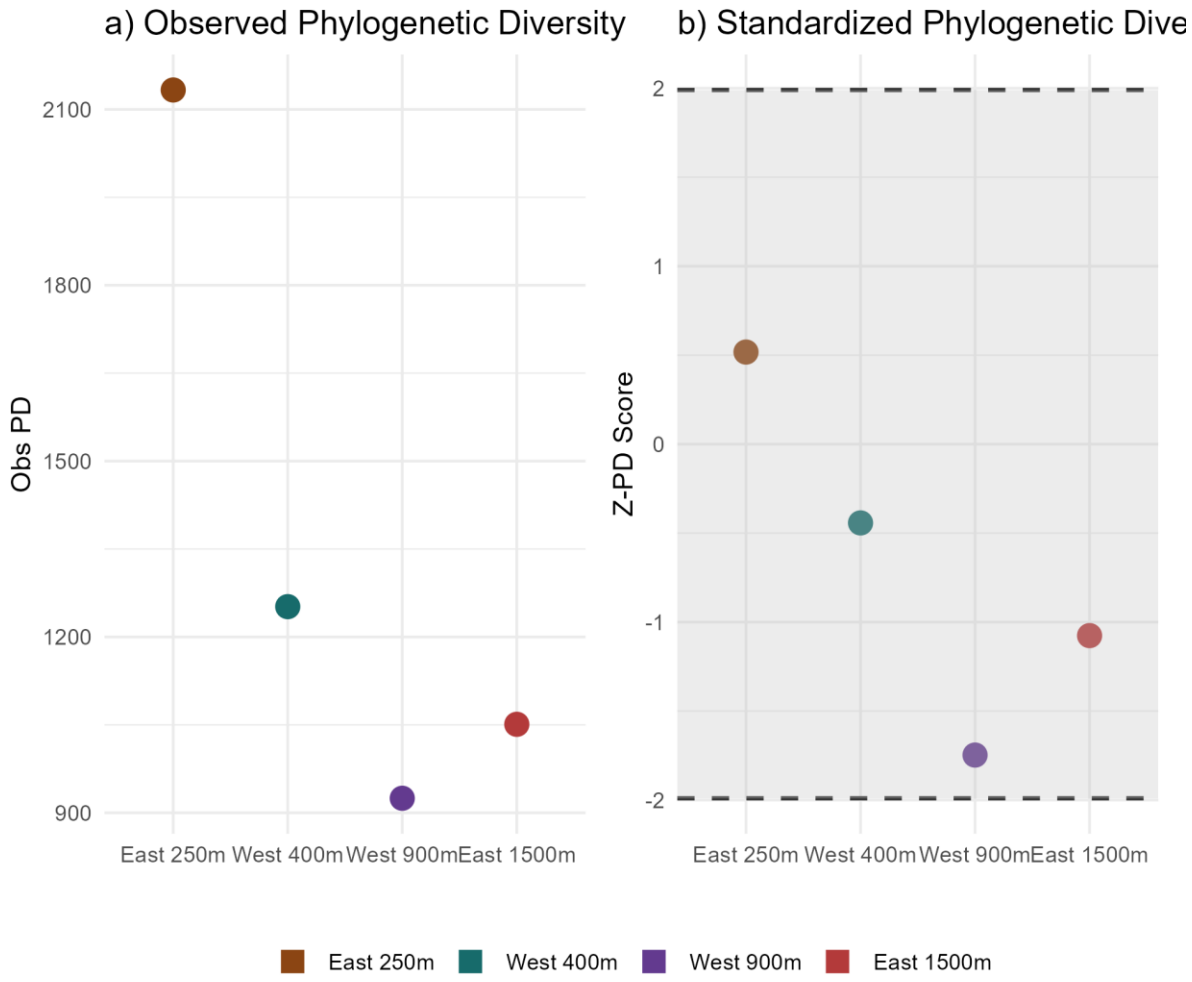


Figure 5: The phylogenetic diversity of communities at different altitude and stratification habitats. (a) Observed Faith's phylogenetic diversity (PD) across altitude and forest stratum. (b) Standardized effect sizes of PD (SES PD) relative to null communities with identical species richness. Dashed lines indicate ±2 standard deviations from the null expectation; values below −2 indicate significant phylogenetic clustering.

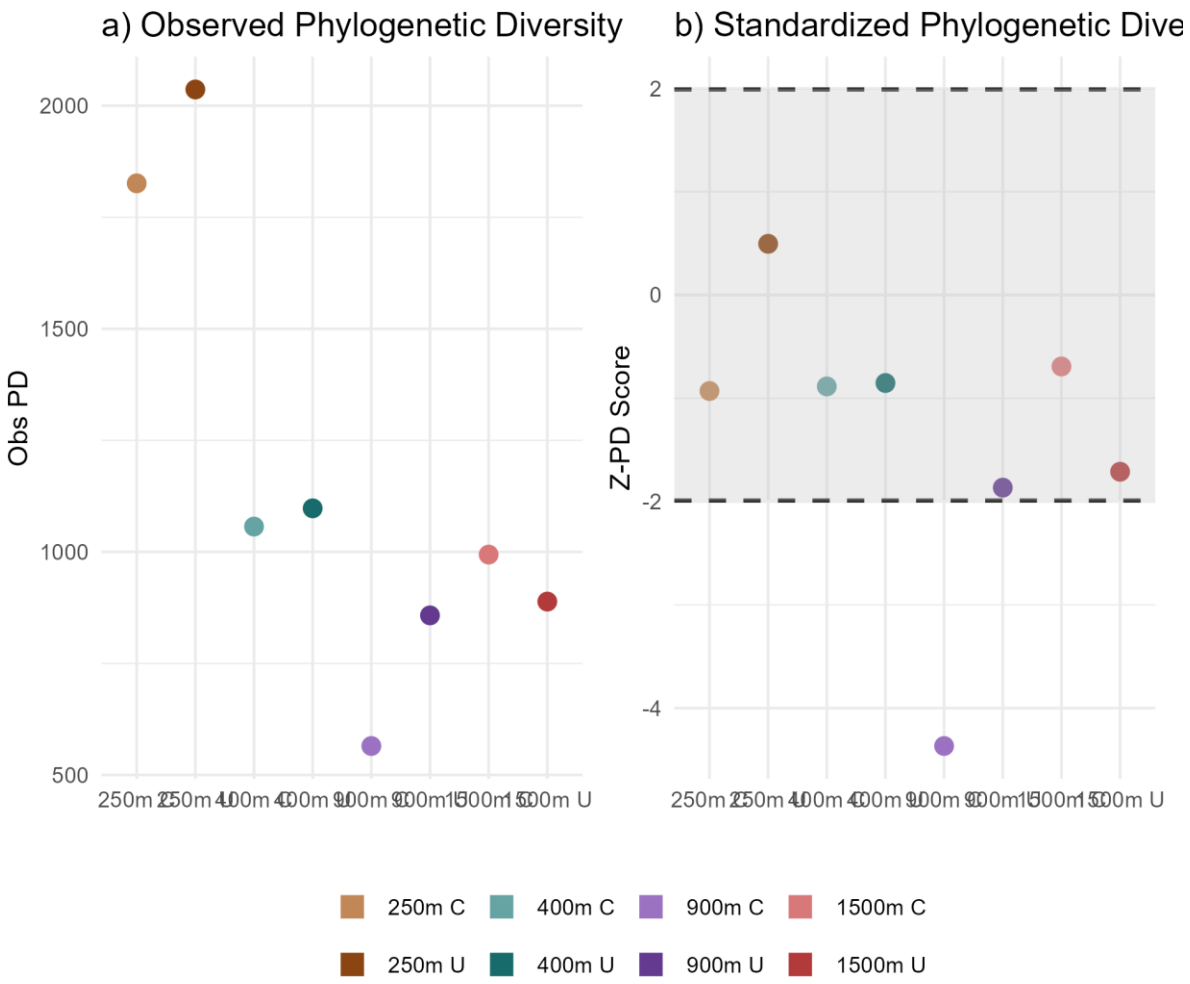


Figure 6: The phylogenetic diversity of communities at different altitude and stratification habitats. (a) Observed Faith's phylogenetic diversity (PD) across altitude and forest stratum. (b) Standardized effect sizes of PD (SES PD) relative to null communities with identical species richness. Dashed lines indicate ±2 standard deviations from the null expectation; values below −2 indicate significant phylogenetic clustering.

Using two different metrics; the NRI (Net Relatedness Index) to analyse the level of deep phylogenetic clustering and the NTI (Nearest Taxon Index) to analyse the level of shallow tip-level phylogenetic clustering among nearest relatives, we observed that phylogenetic community structure and clustering varied across habitat types. At the scale of altitudinal sites, all sites showed deep clustering higher than the null expectation (NRI > 0). Wildsumaco (East 1500m) had the highest NRI

value, suggesting deep clustering in this high-altitude assemblage (Figure 7). Both eastern sites (250m and 1500m) showed negative NTI, suggesting phylogenetic overdispersion at the tips (Figure 8). Durango (West 400m) showed non-significant phylogenetic clustering. Mashpi (West 900m) showed elevated NTI values, indicating higher clustering among closely related taxa here. At the habitat-strata scale, canopy communities generally exhibited higher NRI and NTI values than understory communities, suggesting greater phylogenetic clustering in canopy assemblages (Figure 8). This pattern was most pronounced in the eastern low-canopy assemblage (East 250m), which consistently showed elevated NRI values, and in the western high-canopy assemblage (West 900m), which consistently exhibited high NTI values. In contrast, understory assemblages generally showed weaker and less consistent phylogenetic structure.

There was no consistent relationship between NRI or NTI and altitude or stratification across the East and West Andes, but within Eastern and Western sites, patterns of phylogenetic clustering differed between altitudes and habitat strata. In the East, the low-altitude Yasuni site showed stronger phylogenetic clustering in canopy communities, whereas the high-altitude Wildsumaco site showed the highest overall NRI but negative NTI values. In the West, the mid-altitude Mashpi site showed elevated NTI values, indicating stronger clustering among closely related taxa, while Durango showed weaker and less consistent phylogenetic structure.

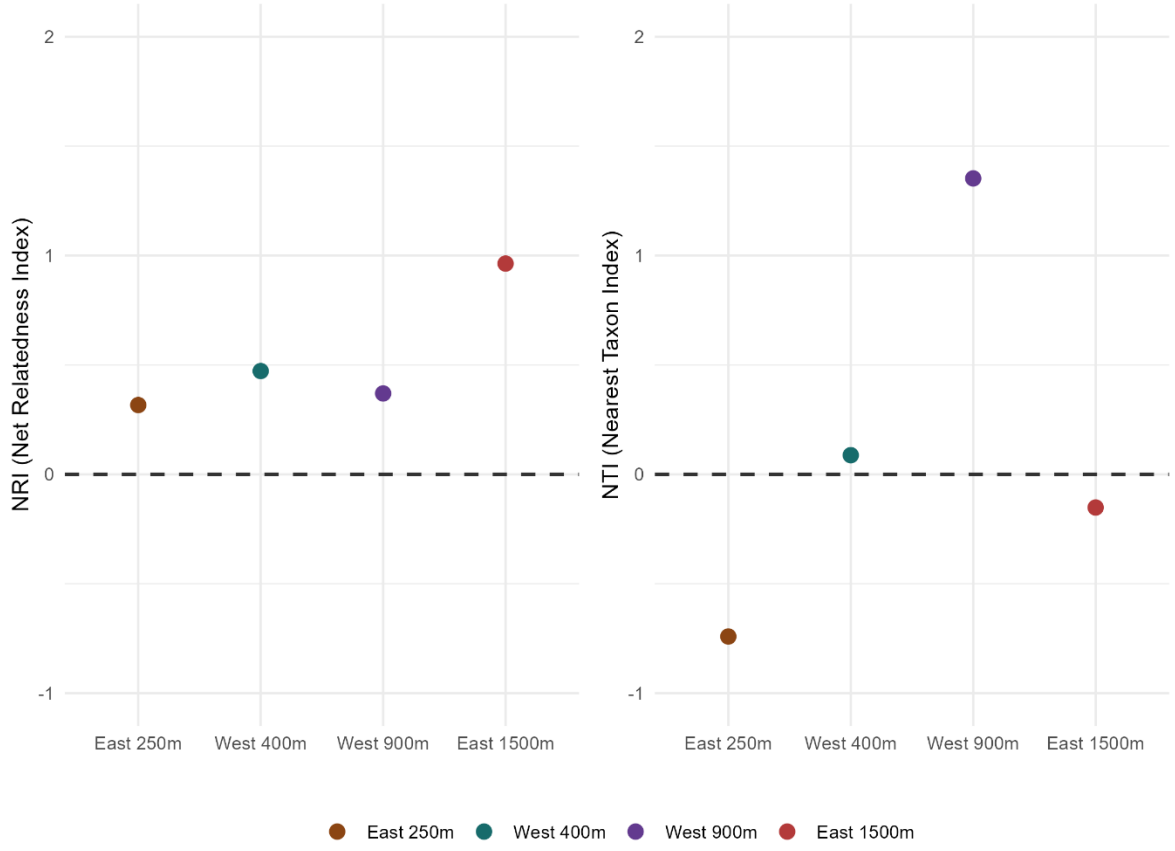


Figure 7: The Net relatedness index (NRI) and the Nearest Taxon index (NTI) values for butterfly assemblages across altitude

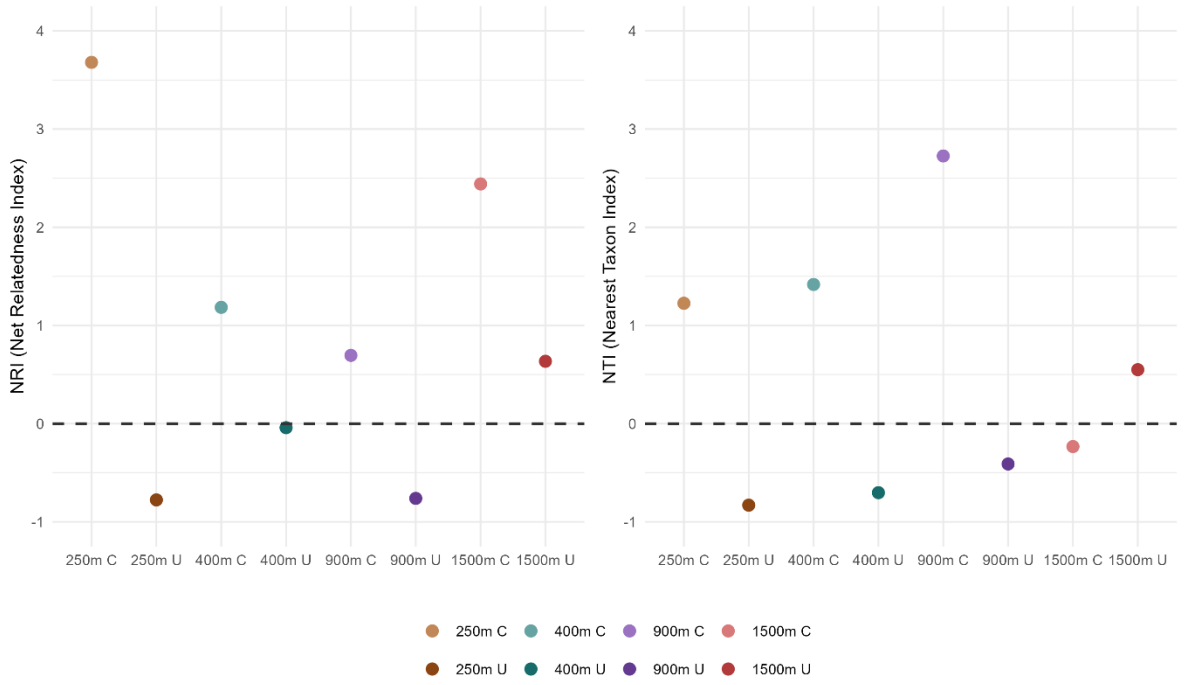


Figure 8: The Net relatedness index (NRI) and the Nearest Taxon index (NTI) values for butterfly assemblages across vertical stratification and altitude.

Community Composition: Beta Diversity

Beta diversity was used to quantify differences in species composition among communities. Communities at different sites were highly dissimilar, with all values above 0.53, indicating few shared species, at the same time, none of the values exceeded 0.86, indicating there was no complete dissimilarity across community pairs (Figure 9). Mid-altitude communities on opposite sides of the Andes (1500 m vs. 900 m) and Mid altitude on the east with low altitude on the east (1500m vs. 400m) were the most dissimilar communities (Sørensen = 0.86 for both). While low-altitude sites (250 m vs. 400 m) and mid- and low-altitude communities on the western Andes (400m vs. 900m) shared the most species (Sørensen= 0.74, Sørensen= respectively 0.53). The latter is likely reflecting the smaller altitudinal gap (500 m), whereas eastern Andes sites with a larger gap (1300 m) shared fewer species (Sørensen= 0.86) (Figure 9).

To examine differences in species composition between the understory and canopy at each site, we calculated Sørensen dissimilarity within and between sites (i.e., across vertical strata) (Figure 10). As

above, communities at different altitudes, which are more geographically isolated, were more dissimilar and shared fewer species. In contrast, species overlap between vertical strata within the same site was higher, as species movement between vertical stratification is more likely than between sites. Consistent with this pattern, within-site community pairs (understory vs. canopy within the same site) showed the lowest dissimilarity values (Sørensen 0.44 - 0.31), indicating the greatest number of shared species (Figure 10). Nonetheless, some degree of dissimilarity was still present between forest strata within the same site, suggesting distinct vertical partitioning of species communities even at a local scale. The mid-altitude sites had greater similarity between canopy and understory communities (Sørensen 0.31-0.36) than the low altitude sites (Sørensen 0.42-0.44) (on both sides of the Andes), likely because vertical distance between canopy and understory is reduced with increasing altitude, creating a less distinct community structure.

There was no consistent pattern of canopy or understory communities being more or less dissimilar between sites. Between the western sites, understory communities were slightly more similar than canopy communities (0.56 vs 0.57), whereas between the eastern sites, there was no difference between canopy and understory community similarity across sites (0.91 vs. 0.91) (Figure 10). The NMDS plot grouped by understory and canopy within altitudes, showed communities are spatially closer within each site, but still exhibit distinct ordination (Figure 11). Here, community overlap is higher between canopy and understory within a site and becomes more distant between sites.

To partition beta diversity into species nestedness and turnover, we used presence-absence data for each species at each site and stratum (Figures 9 and 10). The turnover and nestedness components of beta diversity could each range from 0 (no turnover or no nestedness) to 1 (complete turnover or maximal nestedness contribution). Differences between sites were driven both by turnover and nestedness, but were primarily driven by species turnover, with only a small nestedness component (Figure 9). This is also true when comparing between vertical strata at the same site (Figure 10), indicating that the differences between strata are largely due to unique species inhabiting them, not just differences in species abundances. Furthermore, the observed dominance of turnover in beta diversity is consistent with the alpha diversity patterns being richness-driven (Figure 3 and 4).

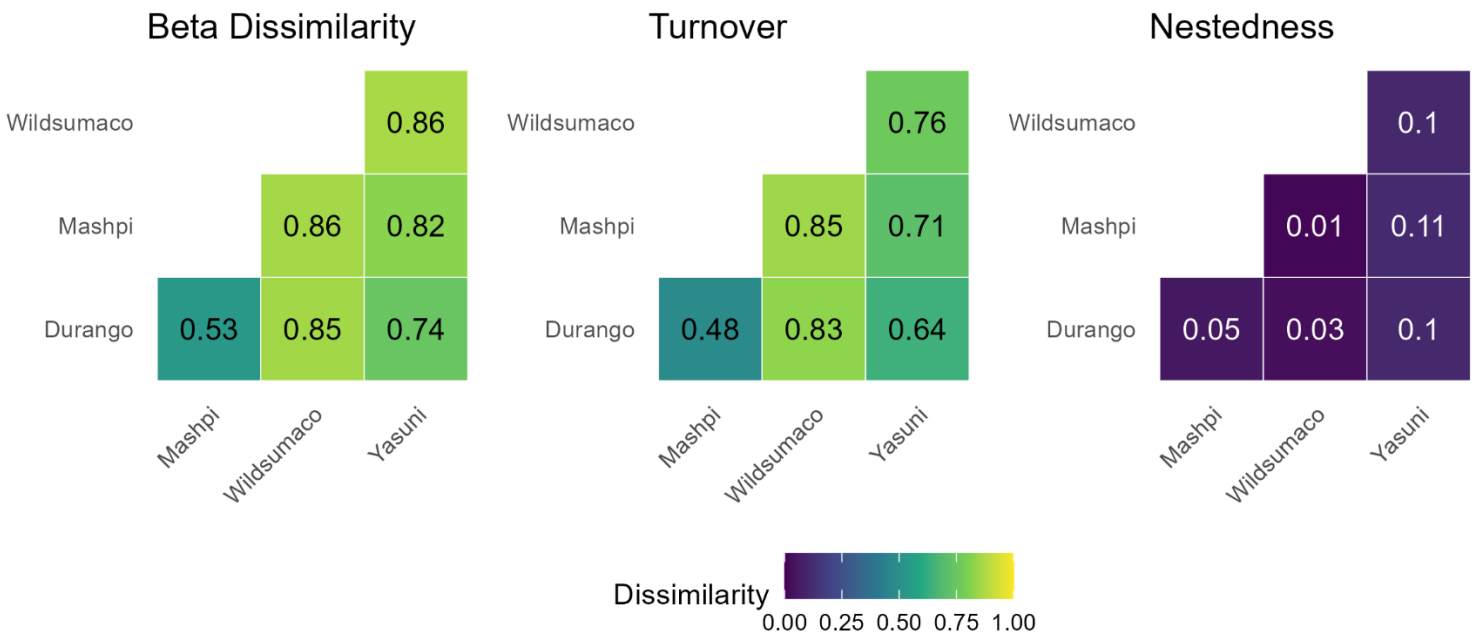


Figure 9: The beta dissimilarity with the turnover (T) and nestedness (N) diversity components for paired combinations of altitude communities. For beta dissimilarity 0 indicates both communities share the same number and abundance of species and 1 indicates there are no shared species between the two. For turnover and nestedness, a value of 0 indicates no turnover or no nestedness and 1 indicates complete turnover or maximal nestedness contribution. Values are coloured in a gradient where dark indicates the lowest values and light indicates the highest values.

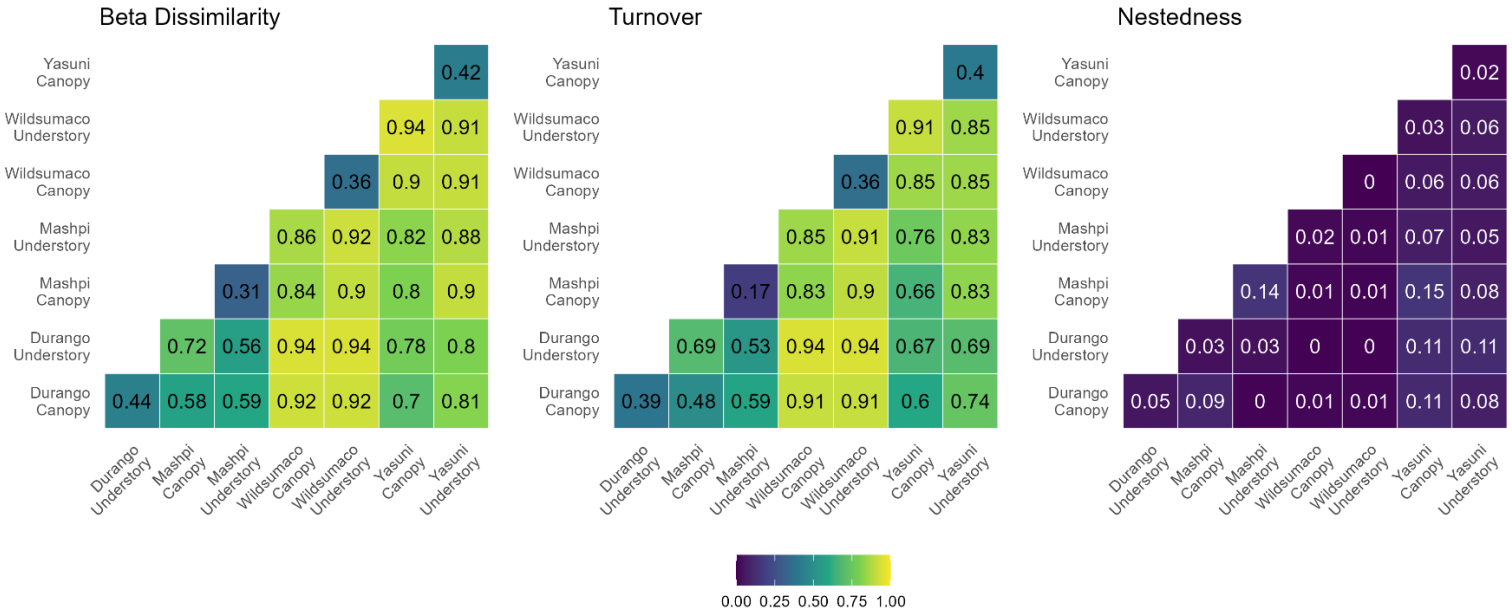


Figure 10: The beta dissimilarity with the turnover (T) and nestedness (N) diversity components for paired combinations of altitude and vertical stratification communities. For beta dissimilarity 0 indicates both communities share the same number and abundance of species and 1 indicates there are no shared species between the two. For turnover and nestedness, a value of 0 indicates no turnover or no nestedness and 1 indicates complete turnover or maximal nestedness contribution. Values are coloured in a gradient where dark indicates the lowest values and light indicates the highest values.

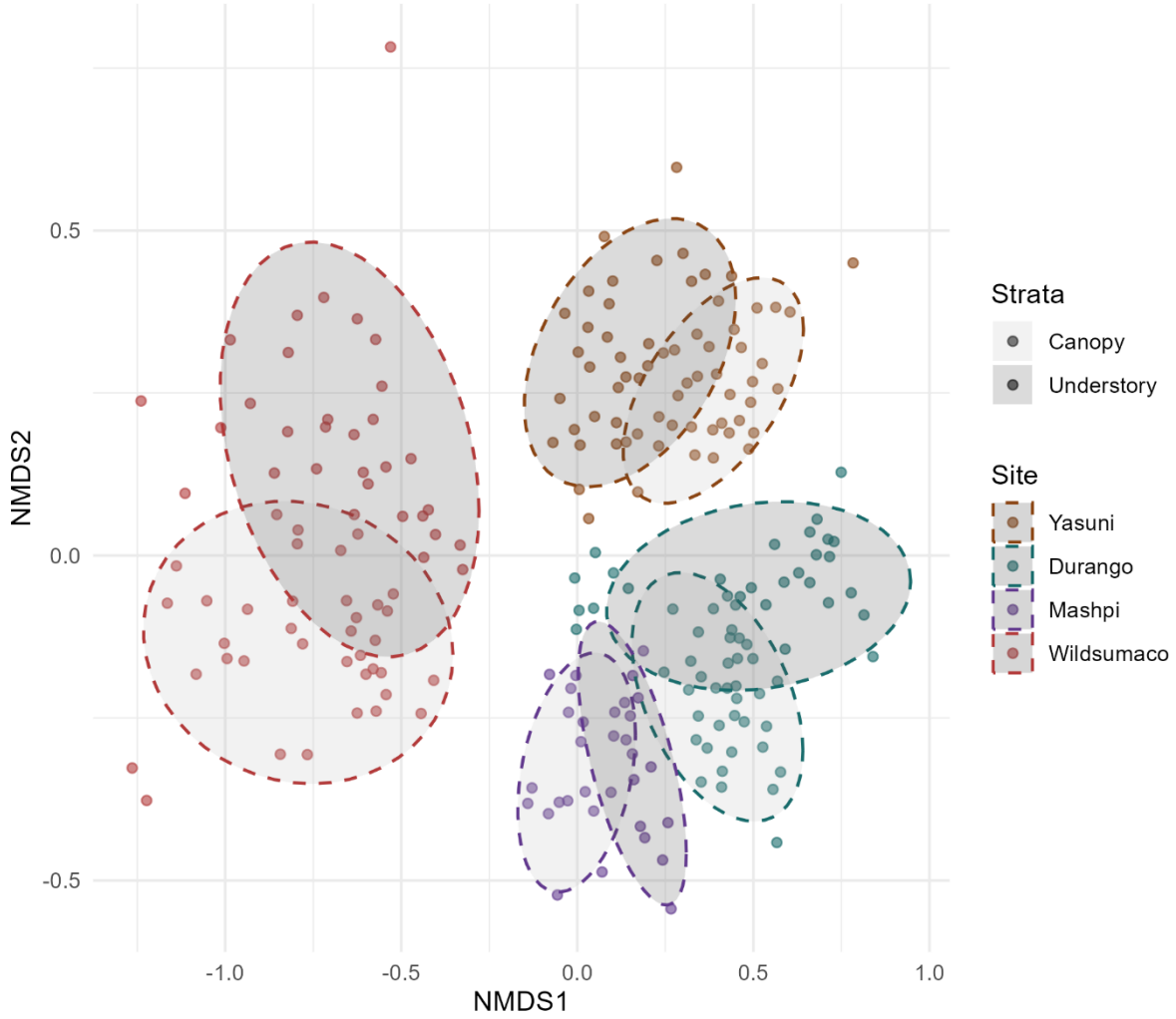


Figure 11: An non-metric multidimensional scaled (NMDS) ordination plot of species grouped by combination of vertical stratification and altitude. The distance between the points (traps) in the plot shows their relative similarity in community. The points closer together are more similar in their community composition than points far apart. The colours represent sites and darker shaded ellipses are the understory communities and lighter shades are the canopy communities. Green indicates the western Andes communities, and orange the eastern Andes and darker colours are low altitude and paler colours mid.

Species distribution: Altitude and Stratification

We produced a scaled distribution value for each species, where 0 indicated all individuals were trapped in low altitude and 1 indicated all individuals were trapped in mid altitude (Figure 12). In both regions, consistent with our previous results, more species were found at low-altitude sites compared

to mid-altitude sites (Figure 12a). The vast majority of species had values of 0 or 1 indicating that they were only found at either the low or mid altitude site. For the eastern and western sites, 9 and 14 species, respectively, had intermediate values between 0 and 1, indicating that they occur across both mid and low altitudes. Most of these still showed a clear trend towards being more abundant at mid or low altitude, while just 2 had values between 0.45 and 0.55 indicating that they were almost equally abundant at the two sites. There were 29 species found in both the east and west. Of these, 21 were consistent in the direction of their relative altitudinal abundance across both sides of the Andes, and 8 species had opposing altitude relative abundances on different sides of the Andes.

For forest stratification preference, the Bayesian hierarchical model produced a binomial distribution ranging from 0 (full understory preference) to 1(full canopy preference). These results indicate the likelihood of occurrence across vertical strata for each species across all traps (Figure 12b). For species found at multiple sites, the strata preference was calculated across all traps at all sites. In contrast to the altitudinal patterns, the distribution of species between the understory and canopy was more intermediate, with few species showing a strong preference for either stratum. Instead, species distributions across vertical stratification were more fluid, with many species being recorded in both the understory and the canopy (Figure 12).

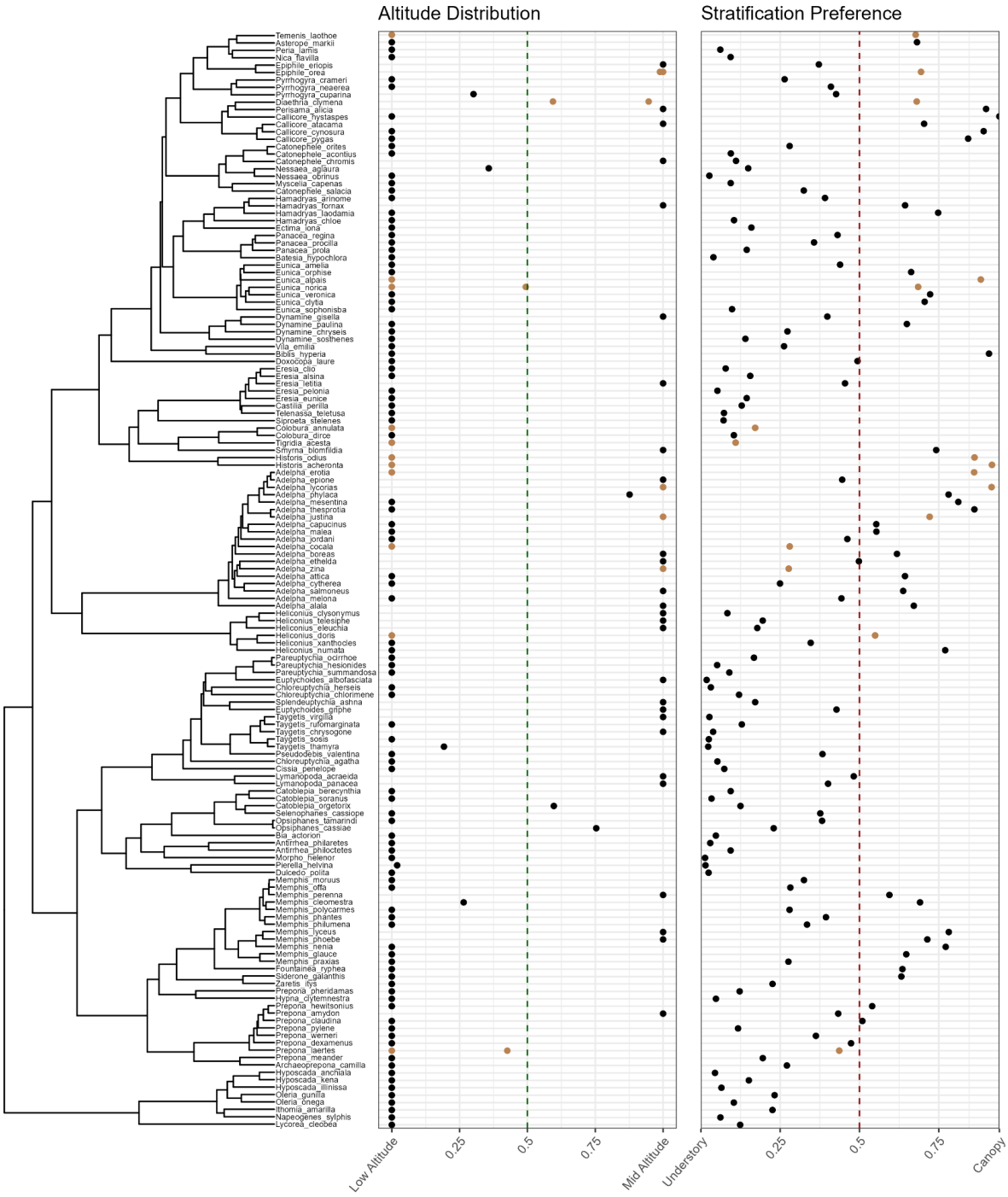


Figure 12: Panel 1: a ladderized cladogram of species. Panel 2: The proportional abundance of species from low to mid altitudes and Panel 3 the stratification preference. The dotted lines indicate where species were found equally in both habitats. The lighter points indicate species found across both sides of the Andes.

2.5 Discussion

This study aimed to characterise Neotropical butterfly communities across four sites at different altitudes on the East and West sides of the Andes and between understory and canopy strata. Our rarefaction curves indicate that sampling was sufficient to capture community composition across sites, allowing robust comparisons of species richness, diversity, and phylogenetic structure. We hypothesized that communities would be most distinct between eastern and western Andes, somewhat distinct across altitudes, and less so between understory and canopy habitats at the same site. These patterns indicate substantial differences in community composition among the sampled sites, which may reflect differences in altitude, geographic separation, or other site-specific environmental factors. However, because each elevation was represented by a single site, and altitude and geographic location were not independently replicated, their relative contributions to community composition cannot be disentangled.

Community patterns across geographic regions and altitude

Alpha diversity showed clear differences between eastern and western Andes communities, consistent with our predictions. These patterns likely reflect the geographic barrier of the Andes, which limits species exchange and reduces shared taxa between subregions (Kattan, 2004). Across altitudes, we observed a consistent decline in species richness and diversity with increasing altitude. Rarefied species richness and Shannon/Hill diversity indices both indicated higher diversity at lowland sites (250–400 m), lower diversity at mid altitudes (900 m), and a slight rebound at 1500 m, though still below lowland levels. This pattern aligns with the hypothesis that harsher, more variable conditions at higher altitude select for specialized species, while lowland habitats support a greater number of generalist species and lineages (Janzen, 1988; Wolda, 1987; Willig & Presley, 2016).

There was higher diversity on the east side of the Andes and in the low altitude on the western side of the Andes. The eastern Amazonian region is influenced by the Amazon basin and is more humid with lush forestation, whilst the western slope of the Andes is more patchy and consists of a range of isolated ecosystems, such as Mashpi (900m) (Ramsay, 2008; Peters et al., 2013; Borchsenius, 1997). There has been a higher level of habitat destruction on the Western slopes, and higher altitudes boast

higher levels of endemic species due to isolated ecosystems and reduction in forest flora available (Ramsay, 2008; Borchsenius, 1997). Differences in altitude on both sides of the Andes is characterised by differences in diversity, where lower altitudes have a higher range of species. Our results coincide with this as low altitudes on both slopes showed higher diversity, compared to the mid altitudes. Further, we also see a notably lower diversity in the western communities, potentially driven by increased endemism, increased habitat degradation and forest patchiness, leading to fewer host plants available for a wider range of species (Tapia-Armijos et al., 2015; Peters et al., 2013).

Beta diversity analysis showed that communities at different altitudes were highly distinct, with limited species overlap, indicating strong environmental filtering along the altitudinal gradient. Lowland sites shared more species across regions than mid altitude sites, likely reflecting more generalist lineages adapted to the less extreme abiotic conditions in the lowlands. Species distribution analyses confirmed that most species are restricted to either low or mid altitudes, with relatively few generalist species occupying multiple altitudinal environments. Together, these patterns indicate that altitude is a major driver of butterfly community composition, mediated by both abiotic conditions and species-specific ecological adaptations.

As Ecuador and Colombia have a particularly high diversity of butterflies (Huertas, B., Le Crom, J. F. & Correa-Carmona, Y., 2022), our results suggest that diversity is maintained by both high species richness at individual sites and differences in community composition. We found low altitude sites had higher species richness, however communities here remained highly dissimilar, where the beta diversity was predominantly driven by species turnover, rather than nestedness. The division of beta diversity into its nestedness and turnover components, revealed the spatial patterns across the sites are driven more by species turnover than nestedness. This suggests that smaller communities, at higher altitudes are not subsets of the larger lower altitude communities but rather are made up assemblages formed through niche specialisation or species replacement. Similar patterns of beta diversity being driven by species turnover have been observed in communities of butterflies in the Eastern Himalayas (Dewan et al., 2022), the Olympus and Rhodopes mountain range in Greece (Kaltsas et al., 2018), and the Eastern Alps in Italy (Fontana et al., 2020). As the beta dissimilarity between communities likely

due to reorganization or local species extinction, this suggests butterfly communities in the Ecuadorian tropics are structured by environmental filtering or spatial sorting (Dewan et al., 2022; Qian et al., 2005). If species turnover is associated strongly with environmental factors, these neotropical communities may be highly sensitive to shifts in climate change, temperature and environment which could disrupt established communities.

Community stratification within sites

Across forest strata, species overlap was higher than between sites, as expected given the lack of geographic isolation between canopy and understory. At one site (400m), there was no difference between understory and canopy community richness. At two of the sites, species richness and diversity were higher in the understory than the canopy (250m and 900m). This contrasts with a previous study, conducted in the Eastern lowland, suggesting higher canopy diversity (Devries et al., 2008), although one of our sites (1500 m on the East of the Andes) did show this trend. Notably, the previous study was conducted on only fruit-feeding butterflies. We were not able to assess the effects of bait type with this data, however we cannot rule out the possibility that using different bait types may impact the variety of species trapped (Salazar- Carrión., et al 2026; Whitworth., et al 2018). Bait type may also impact distribution across vertical stratification, however this is under-researched. Food availability between fruit and carrion is likely to vary between the canopy and understory, where fresh growing fruit is likely to be more abundant in the canopy whilst the understory is where dung, decomposing matter and fallen fruit are concentrated on the forest floor (Hamer., et al 2006). This could therefore drive a difference in fruit and carrion feeding butterflies across vertical stratification.

Our results clearly imply that diversity patterns with forest stratification are not consistent between forest sites. We suggest that low canopy diversity could reflect a combination of ecological constraints, including thermal variability, predation risk, and resource availability in the canopy. Beta diversity analyses revealed partial stratification; while many species were shared between understory and canopy, some level of differentiation persisted, suggesting niche partitioning at smaller vertical scales. Partitioning beta diversity into nestedness and turnover revealed that differences in

community composition were primarily driven by species turnover rather than nested subsets. This dominance of turnover aligns with the richness-driven patterns observed in alpha diversity, indicating that unique species assemblages, rather than nested subsets of common taxa, characterize both altitudinal and stratification gradients (Qian et al., 2005).

The phylogenetic diversity (PD) closely mirrored species richness trends. Lowland sites harbored more distantly related species, while mid altitude sites exhibited greater phylogenetic clustering, consistent with environmental filtering under harsher conditions. Within lower altitudes (250–900 m), canopy communities tended to include more closely related species than the understory, suggesting that canopy habitats may favour particular lineages adapted to more variable conditions. At 1500 m, however, the trend reversed: understory communities were more phylogenetically clustered than canopy communities, possibly reflecting cooler, more stable microclimates in shaded understory habitats that favour specialized lineages (Hazzi et al., 2018). Overall, phylogenetic diversity analyses support the interpretation that altitude and vertical stratification jointly shape butterfly community assembly, influencing both species richness and evolutionary lineage composition.

Phylogenetic clustering analyses revealed widespread deep phylogenetic clustering across assemblages, particularly at higher elevations, suggesting that environmental filtering may play an important role in structuring butterfly communities across different altitudes. However, there was no consistent relationship between NRI and altitude across the eastern and western Andes, suggesting that phylogenetic community structure was influenced by local site conditions rather than altitude alone. The stronger NRI values observed at higher altitudes indicate that these assemblages are composed of lineages that were more distantly conserved than expected by random chance, potentially reflecting shared adaptations to cooler and more variable environmental conditions. However, because NRI does not identify the processes responsible for phylogenetic clustering, this interpretation should be considered as one possible explanation rather than direct evidence of environmental filtering.

Patterns of shallow phylogenetic structure differed between regions. Mashpi (West 900 m) exhibited significant NTI clustering, indicating that closely related taxa co-occurred more frequently than

expected. This may reflect more recent diversification, habitat specialization, or localized environmental filtering within western Andean assemblages. The western Andes are characterized by more patchy, isolated and heterogeneous habitats, which may promote localized adaptation and lineage sorting (Ramsay, 2008; Peters et al., 2013; Borchsenius, 1997). However, as with NRI, elevated NTI alone cannot distinguish between these potential mechanisms or provide direct evidence for more recent diversification. The combination of relatively high NRI and NTI at Mashpi therefore suggests that phylogenetic structure may occur across both deeper and shallower parts of the phylogeny, potentially reflecting multiple ecological or historical influences on community assembly. In contrast, the eastern assemblages, at both middle and low altitudes, exhibited negative NTI values despite positive NRI values, indicating phylogenetic overdispersion among closely related taxa within broadly conserved lineages. This pattern may reflect niche partitioning or competitive exclusion among closely related species, limiting local coexistence of recently diverged taxa while retaining deeper phylogenetic structure.

Interestingly we observed that canopy assemblages were generally more phylogenetically clustered than understory assemblages. These results suggest that canopy communities may be more strongly shaped by environmental filtering, potentially due to the greater thermal variability, solar exposure, and microclimatic extremes experienced in canopy habitats (Scheffers et al., 2017). The combination of elevated NRI and NTI values in some canopy assemblages further suggests that both conserved lineage-level traits and more recent habitat specialization may contribute to community assembly within vertically stratified forest systems. These results evidence that environmental filtering is not only phylogenetically affecting communities across altitudes but is also having a phylogenetic effect at the forest stratification level. Overall, these results indicate that phylogenetic community structure varies not only across altitudinal gradients but also between forest strata, highlighting the potential importance of vertical habitat structure in shaping tropical butterfly assemblages.

Conclusions

Butterfly communities were distinct both across altitudes and between vertical forest strata, suggesting geographical separation and altitude have a clear influence on community composition.

Lowland communities consistently exhibited higher species richness and phylogenetic diversity, while the mid altitude communities were more environmentally filtered and phylogenetically clustered. Vertical stratification further contributed to local community differentiation, though species overlap between canopy and understory was generally high. Taken together, these results highlight the combined influence of geographic isolation, abiotic environment, and vertical habitat structure on Neotropical butterfly communities. Our findings provide a robust framework for understanding species distributions and community assembly, and the species-specific metrics generated here will inform subsequent studies in this system.

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2.7 Supplementary Figures

Supplementary Table 1: The Shannon and Simpsons index, and hill diversity for α- diversity across communities at different altitudes and sides of the Andes. Bold values indicate the highest diversity. The green rows indicate sites from the West side of the Andes and the orange from the East side of the Andes.

Side of Andes	**Altitude**	**Shannon Entropy (H)**	**Simpsons Index (D)**	**Hill Index q0**	**Hill Shannon q1**	**Hill Simpson q2**
West	Low (400m)	3.34	0.94	64	28.22	16.07
	Mid (900m)	3.18	0.93	52	24.01	14.82
East	Low (250m)	**3.95**	0.97	114	**51.85**	30.11
	Mid (1500m)	2.60	0.85	46	13.40	6.74

Supplementary Table 2: The Shannon and Simpsons index, and Hill diversity for α- diversity across altitudes and stratification. Bold values indicate the highest diversity. The green rows indicate sites from the Western andes and the orange from the Eastern andes.

Side of Andes	**Altitude**	**Stratification**	**Shannon Entropy (H)**	**Simpsons Index (D)**	**Hill Index q0**	**Hill Shannon q1**	**Hill Simpson q2**
West	Low (400m)	Understory	2.98	0.91	36	19.78	11.07
	Low (400m)	Canopy	2.98	0.92	42	19.75	11.92
	Mid (900m)	Understory	3.12	0.93	41	22.62	14.92
	Mid (900m)	Canopy	2.68	0.89	29	14.55	9.52
East	Low (250m)	Understory	**3.76**	0.96	76	**42.74**	27.85
	Low (250m)	Canopy	3.45	0.93	72	31.36	14.80
	Mid (1500m)	Understory	2.40	0.83	33	10.99	5.89
	Mid (1500m)	Canopy	2.58	0.88	33	13.25	8.03

3.0 Multispectral reflectance in tropical butterflies across forest stratification and altitudinal range

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Ethics: Museum specimens used in this study were from the Natural History Museum, London. The image data will be provided on the open access museum repository with specimen barcodes included. Fieldwork was conducted with permission for the Ecuadorian government. The majority of captured specimens were released, with only a subset kept for identification and catalogue purposes.

Data Availability Statement: The code and data is available from: https://figshare.com/s/0baac779178440ae524a. The museum specimen images can be found at: (museum repository).

3.1 Abstract

Butterfly distributions and traits are likely shaped by contrasting thermal and light environments that vary vertically within forests and across altitudinal gradients. We investigated the relationships

between wing optical properties, wing size, species occupancy at different altitudinal sites, and preferred vertical position within the forest. Using multispectral image analyses of museum specimens from 148 neotropical nymphalid butterflies, we quantified reflectance across three spectral ranges (ultraviolet, visible and near infrared), visually-scored the presence of iridescent structural colour, and related these to habitat use inferred from trapping data, using phylogenetic comparative methods. Wing colouration differed significantly across strata: visible reflectance declined from the understory to the canopy, indicating darker, more light-absorbing wings in high-light environments. Reflectance across spectral ranges was generally correlated, but the relationship between visible and infrared reflectance was altered by iridescence. Iridescent species showed reduced infrared reflectance for a given visible reflectance, suggesting that structural colours modify heat absorption independently of pigmentation. Our findings suggest that both thermoregulatory and signalling pressures shape the evolution of optical traits across vertical microhabitats, and indicate that nanostructural features can influence wing temperature. More broadly, our results highlight the multifunctional nature of butterfly colouration in tropical forests.

3.2 Background

Animal colour often varies with habitat **[1],** whether in response to variation in the signalling environment, or temperature, or a combination of both **[2-5].** The environment is a key factor in the selection of colour for optimal signalling, as the light environment influences the spectral wavelengths available for effective signalling **[6]**. In many animals, there is a functional overlap and more complex role of colouration, where changes in colouration are primarily selected for camouflage, but also provide a thermoregulatory function **[3, 7, 8]**. Solar irradiance is the primary source of light utilised in signalling and heat for ectotherms. It consists of ultraviolet and visible wavelengths that are important for communication with conspecifics or predators, as these can be visually detected by animals, where detection varies depending on their visual systems. A large proportion of the light hitting the earth's surface is infrared, and while infrared wavelengths can be detected by some animals such as vampire bats (*Desmodus rotundus*) **[9]** and pit-bearing snakes **[10]**, it is not visually perceived by most animals and therefore its reflection or absorption may be selected primarily for thermoregulatory properties

independently of signalling functions, if possible within the constraints of the material and surface reflective properties.

The link between wing colouration, thermoregulation, and habitat distribution remains poorly understood in Lepidoptera and is particularly relevant in tropical forests, where morphological diversity and environmental heterogeneity are high **[11]**. Whilst temperate faunas are well studied, as taxonomy is largely resolved and data widely available, tropical faunas are often neglected as few studies have examined how butterfly colouration in tropical communities relates to microclimatic conditions. For example, López et al. (2024) found differences in optical and thermal properties across vertical forest strata in a small sample of species **[12]**, while Dalrymple et al. (2018) showed that Australian tropical butterflies were more reflective in well-lit environments **[13]**. The existing literature rarely includes broad taxonomic sampling or insights into non-visible optical properties in the environment.

The Tropical Andes in South America are a biodiversity hotspot for insects and provide an ideal setting for studying these patterns **[11, 14-16].** Tropical habitats are often considered climatically stable, but they encompass a wide range of macro- and microclimatic conditions. Their extensive altitudinal gradients produce marked variation in temperature, humidity, and solar exposure **[15-17]**. Within each altitudinal environment, the vertical forest stratification further creates distinct microclimates **[18, 19]**. The understory is thermally buffered, cooler, darker and has lower solar exposure (less light reaching the ground level), and the canopy is warmer, more variable in temperature and more irradiated **[15-19].** While microclimate may determine the broad selective pressures on colour, the optical properties of the absorption surface may shape habitat preference across differing microclimates in vertical forest stratification, by mediating thermal tolerance. Butterflies are highly diverse in the neotropics **[20, 21]**, occupy varied niches within these environments, and exhibit a striking array of colours. They are therefore subject to selection for traits that enable them to cope with environmental heterogeneity **[20-24].** Understanding the mechanisms underlying thermal adaptation is important for predicting how Neotropical butterflies may respond to future climatic shifts and how colour morphology is selected in tropical habitats.

Butterfly wings are less thermally stable than the thorax, and various thermoregulatory mechanisms are utilised to regulate temperature of the wings separately from the body, including convective heating/cooling (via haemolymph flow), and detection of solar radiation with subsequent basking or shade seeking **[25-27].** Wings are also used for thoracic temperature regulation, such as in basking, through the direction of light onto the body and through hemolymph flow between the body and wings **[28]**. These active thermoregulatory mechanisms often exploit radiative cooling/heating of wing surfaces via absorption or emission and reflection of infrared light **[28]**. Importantly, aside from active thermoregulation, the radiative properties of the wing surface are dependent on the wing material properties and are impacted by pigmentary and structural colouration **[28]**. Perceivable colours such as visible pigmentary and structural colour are typically under selection for communication, yet infrared optical properties emerging as a by-product of the wing material properties, can have a thermoregulatory role, independent of the visible signal **[29-32]**. Studies evidencing this in butterflies are only available for very few species, (e.g., *Colias sp.*) **[30-32]**, but there is the potential for interactions between colouration and thermoregulation across a wider range of butterfly species. Secondary thermoregulatory effects of visible colouration could therefore influence butterfly habitat selection through trade-offs between signalling and infrared-driven thermoregulatory constraints.

Melanic colouration can influence thermal balance and environmental tolerance, potentially shaping habitat selection. For example, at low temperatures, dark individuals may heat up faster at a given level of solar radiation, known as thermal melanism **[5, 7, 32-35].** This has been observed in a range of insects, reptiles and birds and is dependent on the species morphology and environmental conditions **[5, 22, 34-37].** In butterflies, studies have observed a correlation between wing temperature and pigmentation, where darker pigmentation is responsible for increased absorption of heat **[25, 31, 32, 36, 37]**. Indeed, darker wings have been linked to cooler habitats in some species of butterflies, including Monarch (*Danaus plexippus),* Small Apollo *(Parnassius phoebus)* and Clouded Yellow (*Colias sp.*)**[22, 31, 32, 38, 39].** Increased heat absorption can be especially beneficial for warming up in cooler environments or for maintenance of wing temperature in species that occupy larger

biogeographical ranges with more variable thermal environments **[22, 23, 36, 31].** Melanin pigments also absorb harmful ultraviolet wavelengths, which are converted into heat, providing increased protection against damage **[40-42].** This increased UV protection may be beneficial in environments where solar irradiation is higher (e.g., in high altitude tropical mountains) **[43, 44].**

Beyond pigmentation, butterfly wings have colour-producing nanostructures such as thin-film multilayers that are responsible for the striking iridescence, often blue, seen in many species. Structural colour arises from physical light interference within multilayered chitinous nanostructures which also affect infrared reflectivity **[45, 27]**. The architecture of these nanostructures within wing scales determines the optical properties, as seen in previous studies where the efficacy of radiative cooling of butterfly wings was determined by the thickness of chitinous nanostructures [**45, 27]**. The optical properties of thin-film multilayers, responsible for iridescent colour, are often overlooked in the infrared range of the light spectrum **[45, 46].** Nanostructures are multifunctional and may not only be used for visual signalling but could also have secondary thermoregulatory functions by influencing reflectance and absorption of heat from the wing surface. Melanin embedded within these structures further influences how light, especially infrared, interacts with the wing **[46, 47].** This relationship between pigmentary and structural colour exists in birds, where the precise arrangement of melanin in feathers can affect a bird's visible appearance, which is important in signalling, but also the structural heat absorption properties of feathers **[48-51]**. Similarly, for butterflies, the same complexities arise with signalling, crypsis and thermoregulatory properties of their colouration.

Here, we examine ultraviolet (UV), visible (VIS), and infrared (IR) optical properties (200–1040 nm) of tropical nymphalid butterfly wings in relation to their occurrence in low or mid-altitude forest and their preference for flying in the canopy or understory. We compared species with and without true structural iridescence to determine whether structural colour significantly impacts optical properties across the different parts of the spectrum. Using museum specimens representing 148 species with available trapping data to determine altitude distribution and vertical habitat preference, we evaluate how wing reflectivity varies across habitats. Our comparative approach provides insights into the thermal and optical diversification of tropical nymphalids across forest habitats that differ in thermal

and light environment on both broad and narrow spatial scales.

3.3 Methods

Trapping Sites

Data on butterfly communities was obtained from monitoring programmes carried out across four sites in Ecuador at mid (900m and 1500m) and low (250m and 400m) altitudes, on the east and west sides of the Andes (Supplementary Fig.1). The sites are as follows: Yasuni National Park, a humid tropical rainforest in Pastaza and Orellana provinces (250m, 00430S, 80093W), Wildsumaco Lodge, a humid, cloud forest in Napo province (1500m, -.674638889 S, -77.60886111 W, 1500m), Mashpi Rainforest Reserve, a rain/cloud forest in Pichincha province (900m, 0957 N, 785237 W) and El Durango, a very wet tropical rainforest in Esmeraldas province (400m 10053 N, 783619 W).

These sites were selected for this study as they encapsulate a broad altitudinal range (from 250 to 1500 m) that is known to represent different butterfly assemblages and has been shown to encompass distinct thermal environments **[16]**, and data across these sites were collected under the same trapping protocols. Trapping data were provided from fieldwork from 2014 to 2017. A total of 32 Van Someren Rydon bait traps were paired in the understory (0.37– 0.92 m) and the canopy (11–27.3m), and each pair were spaced between 47–127m from one another **[52]** (Supplementary Fig. 1). Traps were baited with either fermented banana or rotted shrimp to increase the range of bait-feeding butterflies sampled **[53-55].**

Altitude and Vertical Stratification Distributions

Trapping data were processed in Excel and R v4.3.0. Singletons (69 data points represented by only a single occurrence across all sites) were excluded and removed from the dataset due to unreliable frequency estimates. We used the trapping data across the four sites to obtain proxies for species distributions. To account for unequal sampling effort among sites (2014–2017), species counts were standardised as relative abundances per site (species count divided by total individuals captured). Relative abundances were used to quantify altitudinal distributions using a scaled index, a/(a+b), where *a* and *b* represent relative abundance at low and mid altitude, respectively. As there was a clear

altitudinal separation, with most species being present at only one site (Supplementary Fig. 2a), the altitudinal distribution was used as a categorical variable in further modelling. Values < 0.49 were assigned as low-altitude and values > 0.51 were assigned as mid-altitude. For 29 species found across both sides of the Andes, relative abundance was calculated separately for eastern and western sites. Of these 29 species, 21 species had the same altitude occupancy on both sides of the Andes, while 8 species had different altitude distributions on different sides of the Andes and were removed for subsequent analysis. Vertical stratification (understory vs. canopy) was estimated using a hierarchical Bayesian model implemented in BayesPref v1.0 on paired understory–canopy trap data **[56]**. Because individuals frequently occur in both strata, unlike altitudes, this approach provides a more robust preference estimate. Occurrence was modelled as a binomial response (1 = canopy, 0 = understory) using 4,000 MCMC iterations, a 1,000-iteration burn-in, and default priors. Species-level posterior preferences were averaged across traps for each species (Supplementary Fig. 2b) and this vertical stratification value was used as a continuous variable in further modelling.

Museum Specimens, Imaging, and Image Processing

We digitally photographed 148 dry, pinned male butterfly specimens from the Natural History Museum London (NHMUK). Males were used because trapping data primarily represented males, which are more likely to be attracted to traps than females. Species represented in the trapping data were selected, prioritizing specimens from Ecuador to minimize regional variation; when unavailable, west Colombian specimens were used. Subspecies were not separated due to high taxonomic uncertainty and frequent misidentification in some Neotropical groups (see Species List in Supplementary Table 1).

Digital photographs were taken using a Nikon D700 DSLR with a Rayfact UV-105mm F4.5 lens and a converted full-spectrum (300nm – 1500nm) Nikon SB500 flash unit, to ensure that illumination would reach a broader range of UV to IR wavelengths and enable our camera filters to capture these. Images were captured inside a light-controlled photography box. Three filters isolated UV, VIS and IR wavelengths: U-filter (320–380 nm; UV only), UV/IR-cut (400–680 nm; VIS only), and IR-pass (≥685 nm; IR only). Each specimen was photographed dorsally and ventrally in all three spectra (six

images per specimen). The presence or absence of iridescence was determined through visual inspection, for dorsal wings only; ventral iridescence was difficult to determine, as it is rare and typically minimal.

Images were processed in ImageJ using MicaToolbox **[57, 58]**. A ColourChecker Nano served as the calibration point. Reflectances of the greyscale standards were measured with an Ocean Optics spectrometer (Supplementary Fig. 3a), and the percentage reflectance for UV (200–400 nm), VIS (400–780 nm) and IR (780–1040 nm) ranges was calculated in the PAVO R-package **[59]**, relative to a Spectralon white standard (Supplementary Fig. 3b). These values were used to calibrate multispectral images in ImageJ (VIS: *94,65,47,24,12,5*; IR: *98,59,39,20,11*, 6; UV: *50,36,29,17,10,6*).

Raw images were imported into ImageJ, scaled to 366.20 px cm^{-1}, and converted into multispectral stacks (Fig. 1). Because images were calibrated by spectral range and captured with appropriate filters, the resulting summed RGB values corresponded to the total reflectance within the camera-channel sensitivity for UV, VIS or IR. Mean RGB reflectance and pixel area were extracted for three regions of interest (ROI, Figure 1) per wing using MicaToolbox. Dorsal and ventral RGB reflectance values were standardized to 8-bit (/255), weighted by ROI size, and summed to obtain total reflectance per spectral range for each species. Because the reflectance values were positively skewed, the final summed reflectance values were log-transformed for statistical analyses. Wing size was also measured from these calibrated images, by scaling the photos by pixel area in ImageJ.

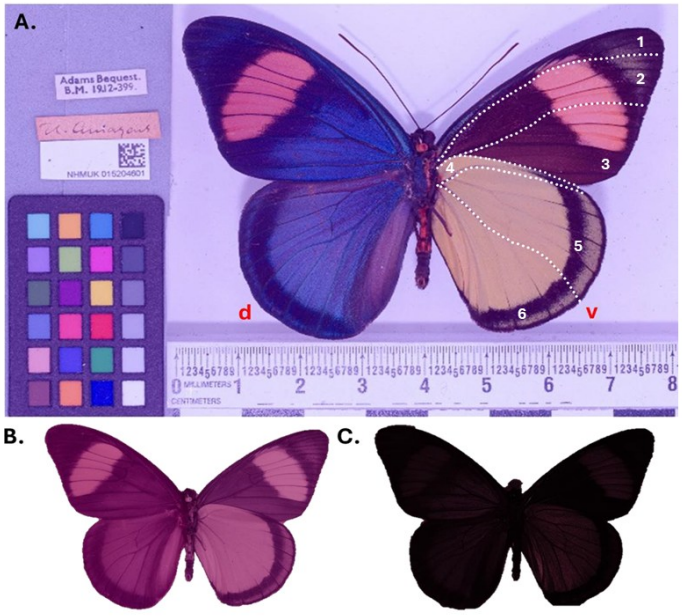


Figure 1: Composite multispectral images of *Batesia hypochlora* (Natural History Museum, UK) edited to show dorsal (d) and ventral (v) sides: (A) VIS reflectance, (B) IR reflectance, and (C) UV reflectance. Dotted white lines indicate the ROI segmentation. Images were captured with the same specimen configuration, including labels, colourchecker, and ruler. IR and UV images appear pinker due to greater sensitivity of the red camera sensor to these wavelengths.

Phylogenetic Comparative Analysis

We used a published phylogeny of 2,880 Nymphalid species (~45% of known diversity) **[60]**. The tree was pruned to include only the 168 species in our dataset. For taxa missing from the phylogeny, 18 species were substituted with the closest related congeners. Twenty species lacking phylogenetic data or suitable substitutes were removed. The final pruned and rooted tree contained 148 species (see Species List in Supplementary Table 1). For initial data exploration, we tested for phylogenetic signal in predictor and response variables; vertical stratification, wing size and reflectance, using the caper package **[61]**. Phylogenetic generalized least squares (PGLS) models were run separately for dorsal and ventral surfaces to investigate dorso-ventral and spectral category correlations. For PGLS models on each spectral range, model selection included size, altitude distribution and stratification preference as predictors; models of IR and UV reflectance also included VIS reflectance. As reflectance was calculated as the sum of the RGB values, here a low visible reflectance would indicate duller, darker colours and a high visible reflectance would indicate brighter or lighter colours. The

best supported models were selected using stepwise selection based on model fit and AIC, retaining the most parsimonious model supported by the data. Terms were removed where their inclusion did not improve model fit or was not supported by AIC, while biologically relevant predictors were retained where supported.

3.4 Results:

Phylogenetic Signal of Morphological Traits

Phylogenetic signal (Pagel's λ) was moderate to high for both predictor and response variables, though confidence intervals were wide and often spanned low to high values (Supplementary Table 2). Despite this uncertainty, no traits were consistent with pure Brownian motion (λ = 1), with all estimates < 1. Trait mapping across the phylogeny (Fig. 2) showed that VIS colouration was more conserved in some subfamilies (e.g. Charaxinae, Heliconiinae) than others (e.g. Satyrinae, Biblidinae, Nymphalinae). Stratification preference was generally less variable, with Satyrinae, Nymphalinae and Danainae consistently associated with lower strata, while other subfamilies exhibited greater within-clade variation. Across the phylogeny, dorsal VIS, IR, and UV reflectance covaried, such that species with high VIS reflectance also showed higher IR and UV reflectance (Fig. 2).

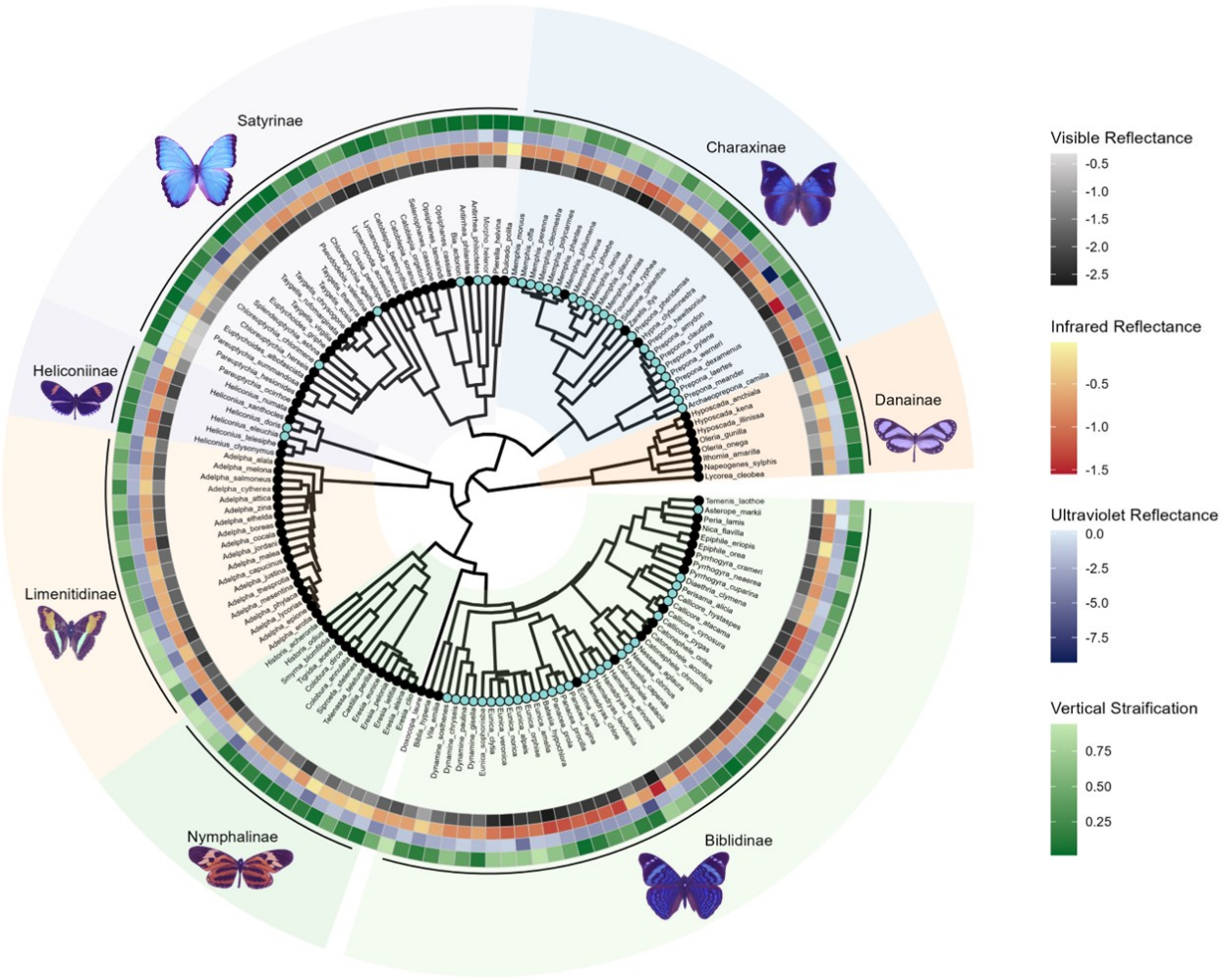


Figure 2: Phylogenetic tree of 148 nymphalid species. Tips are coloured by iridescence (blue = present, black = absent). Concentric rings show dorsal reflectance (log-transformed) and vertical stratification, VIS (black = low, white = high), IR (red = low, yellow = high), UV (dark blue = low, light blue = high), and stratification preference (dark green = understory, light green = canopy). Subfamilies are indicated, and a representative species is shown.

Dorsal–ventral wing relationships:

The PGLS models showed positive correlations between dorsal and ventral reflectance across all spectral ranges. All correlations deviated from a 1:1 trend (Supplementary Fig. 4) and dorsal reflectance was generally lower than ventral reflectance, across all parts of the spectrum, which is consistent with observations that ventral sides are often paler. Correlations were strong in the visible (estimate = 0.782, $p < 2.2\times10^{-16}$, R^2 =0.56) and IR (estimate = 1.020, $p < 2\times10^{-16}$, R^2 = 0.68) ranges. UV reflectance was also correlated but very weakly (estimate = 0.229, p = 0.019, R^2= 0.02)

(Supplementary Fig. 4). This suggests UV reflectance had a greater level of independence between dorsal and ventral surfaces than other parts of the spectrum, but could also be due to greater noise in the UV reflectance estimates due to the reduced sensitivity of our set-up in the UV part of the spectrum.

Spectral reflectance and iridescence:

PGLS models testing correlations between VIS and UV/IR reflectance showed consistently positive relationships (IR–VIS dorsal: estimate = 0.505, $p < 2\times10^{-16}$; IR–VIS ventral: estimate = 0.479, $p < 2.2\times10^{-16}$; UV–VIS dorsal: estimate = 1.137, $p = 8.0\times10^{-6}$; UV–VIS ventral: estimate = 2.174, $p < 2\times10^{-16}$) (Fig. 3a–d) . IR and UV reflectance were not significantly correlated dorsally (estimate = 0.019, p = 0.210), but were positively correlated ventrally (estimate = 0.094, $p = 6.6\times10^{-12}$; Fig. 3e, f), supporting the idea that dorsal UV reflectance evolves more independently. Comparisons between species that did or did not possess dorsal iridescence showed no significant differences in VIS (estimate = –0.079, p = 0.411) or UV reflectance (estimate = –0.029, p = 0.926; Fig. 4a,c). IR reflectance, however, was significantly lower in species with iridescence (estimate = –0.233, $p = 4.1\times10^{-5}$; Fig. 4b).

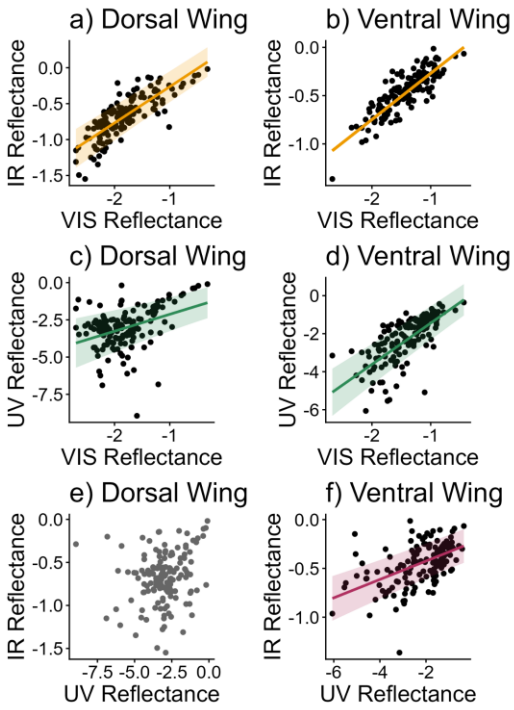


Figure 3: Spectral wing reflectance relationships on the dorsal and ventral surfaces. (a,b) VIS versus IR, (c,d) VIS versus UV, and (e,f) IR versus UV reflectance. Lines show conditional effect, model predictions where the correlation was significant; no significant correlations (e) are in grey. Reflectance values are log-transformed RGB pixel measurements for each species.

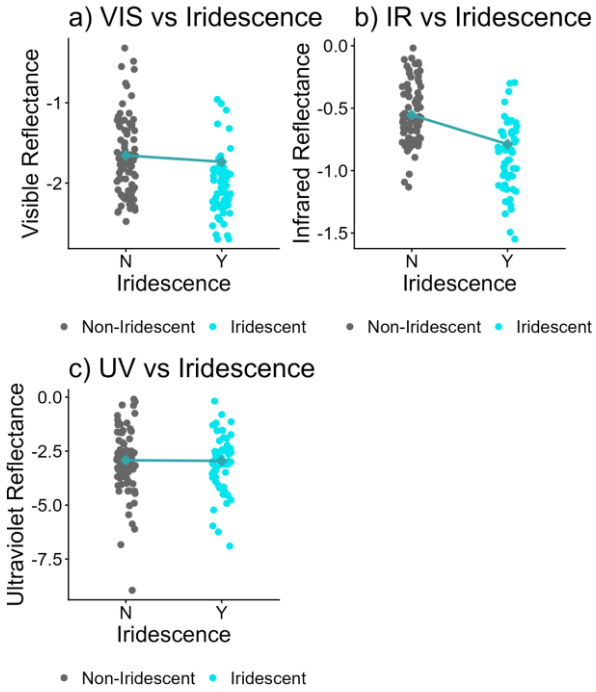


Figure 4: Dorsal reflectance for iridescent and non-iridescent species. Mean values and lines are predicted conditional effects from PGLS models: (a) VIS, (b) IR, and (c) UV reflectance versus iridescence. Observed reflectance values are log-transformed RGB pixel measurements for dorsal.

The effects of altitude, stratification and wing size on reflectance:

Whether a species was found at middle or low altitude sites had no significant effect on dorsal or ventral reflectance in any spectral category (Table 1). VIS reflectance on both wing surfaces was significantly influenced by species stratification preference and size (Table 1a,b). Here, reflectance was higher in understory species and decreased with increasing canopy preference (Fig. 5a,b). Smaller species reflected more visible light than larger species (Fig. 5c,d). IR reflectance showed different patterns dorsally and ventrally (Table 1c,d). Only ventral reflectance decreased slightly in canopy species (Fig. 5f), while size significantly affected both dorsal and ventral surfaces, with smaller species reflecting more IR than larger species (Fig. 5g,h). UV reflectance was generally unaffected by

stratification, and dorsal reflectance was not influenced by size (Table 1e,f; Fig. 5i–k). Ventral UV reflectance, however, decreased with increasing size (Fig. 5l), as seen in VIS and IR ranges. The presence or absence of dorsal iridescence was not significantly predicted by altitude, canopy preference or size (Table 1g).

Table 1: PGLS predicting wing reflectance and iridescence for dorsal and ventral reflectance in VIS (a,b), IR (c,d), and UV (e,f) spectra, and a PGLM on the presence or absence of iridescence (g). Significant predictors or interactions are shown in bold.

a) Dorsal Visible reflectance~					*b) Ventral Visible reflectance ~*			
Coefficients	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**	**Estimate**	**Std. Error**	**t-value**	**Pr(>|t|)**
(Intercept)	-1.369	0.249	-5.488	1.8e-07	-1.081	0.164	-6.606	<0.001
Altitude	-0.003	0.067	-0.046	0.963	0.020	0.067	0.302	0.763
Canopy	**-0.366**	**0.143**	**-2.553**	**0.012**	**-0.337**	**0.131**	**-2.564**	**0.011**
Size	**-0.130**	**0.058**	**-2.228**	**0.028**	**-0.139**	**0.048**	**-2.879**	**0.005**
R^2: 0.059, λ: 0.837					R^2: 0.076, λ: 0.559			
c) Dorsal Infrared Reflectance ~					*d)Ventral Infrared reflectance ~*			
(Intercept)	-0.394	0.117	-3.356	0.001	-0.304	0.086	-3.516	0.001
Altitude	-0.001	0.046	-0.021	0.983	0.009	0.038	0.238	0.812
Canopy	-0.179	0.091	-1.976	0.050	**-0.159**	**0.073**	**-2.189**	**0.030**
Size	**-0.109**	**0.033**	**-3.304**	**0.001**	**-0.070**	**0.026**	**-2.671**	**0.008**
R^2: 0.077, λ: 0.598					R^2: 0.058, λ: 0.516			
e) Dorsal Ultraviolet reflectance ~					*f) Ventral Ultraviolet reflectance ~*			
(Intercept)	-2.526	0.437	-5.776	4.5e-08	-1.728	0.486	-3.560	0.001
Altitude	-0.158	0.266	-0.594	0.554	0.010	0.215	0.045	0.964
Canopy	-0.089	0.476	-0.187	0.852	-0.270	0.413	-0.653	0.515
Size	-0.239	0.157	-1.521	0.130	**-0.312**	**0.149**	**-2.089**	**0.039**
R^2: 0.015, λ: 0.172					R^2: 0.040, λ: 0.240			
g) Presence/Absence of Dorsal Iridescence~								
(Intercept)	-0.796	0.787	-1.011	0.312				
Altitude	-0.007	0.250	-0.030	0.976				
Canopy	0.453	0.567	0.799	0.425				
Size	0.042	0.235	0.179	0.850				

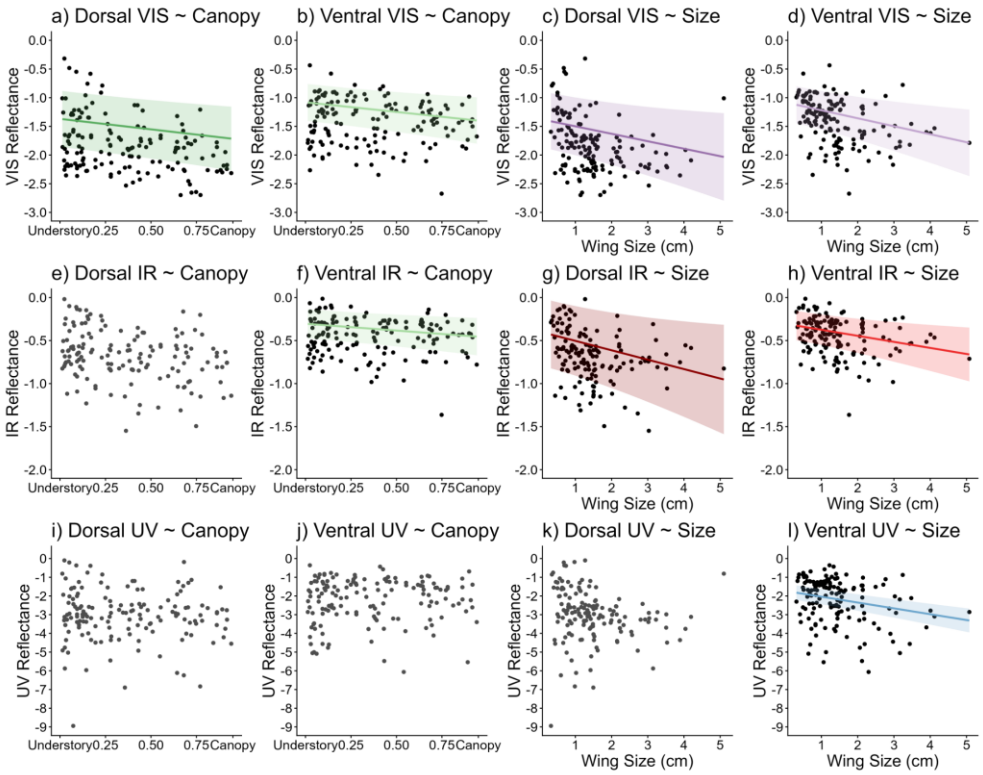


Figure 5: Significant coefficients from PGLS models (Table 1). Panels show relationships between: vertical stratification and VIS reflectance (a,b), size and VIS reflectance (c,d), IR reflectance and canopy (e,f), IR reflectance and size (g,h), and UV reflectance with canopy (i,j) and size (k,l). Non-significant variables from Table 1 are shown in grey for comparison. Points show raw observed data; lines are the predicted conditional effects, and ribbons show the predicted 95% confidence intervals.

Infrared – visible reflectance relationships across stratification, and size:

To assess which factors influenced IR reflectance independently of VIS reflectance, we fitted a full model of IR reflectance including VIS reflectance, stratification preference, size, and iridescence (Table 2). Ventral VIS reflectance was the only significant predictor of ventral IR reflectance (Supplementary Table 3). For dorsal wings, IR reflectance was significantly influenced by size and the interaction between VIS reflectance and iridescence (Table 2). While VIS reflectance was highly correlated with IR (Supplementary Table 4), the inclusion of size, stratification, and iridescence captured the nuanced effects on IR reflectance ($R^2 = 0.67$; Table 2). Size remained negatively

correlated with IR reflectance, showing that this relationship cannot solely be explained by the correlation with VIS reflectance (Table 2).

There was a significant interaction of VIS reflectance and iridescence, effecting IR reflectance. Higher VIS reflectance was consistently associated with higher IR reflectance (Fig. 6), indicating that visibly darker species reflected less IR and paler species more. Interestingly, this relationship differed between iridescent and non-iridescent species, where non-iridescent species had a slope of 0.42, whereas iridescent species had a steeper slope of 0.59 (Table 2; Fig. 6). Thus, at lower VIS reflectance, iridescent species reflected less IR than non-iridescent species. At higher VIS reflectance, the groups converged in IR reflectance (Fig. 6).

Table 2: PGLS model coefficients predicting dorsal IR reflectance. Significant predictors or interactions are shown in bold.

Infrared Reflectance~ VIS Irid + Canopy + Size*				
Coefficients	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	0.195	0.098	1.988	0.049
VIS	**0.416**	**0.040**	**10.437**	**0.000**
Iridescence	0.159	0.124	1.284	0.201
Canopy	0.072	0.057	1.269	0.207
Size	**-0.055**	**0.022**	**-2.545**	**0.012**
VIS: Iridescence	**0.169**	**0.064**	**2.649**	**0.009**
			R^2: 0.6651, λ: 0.705	

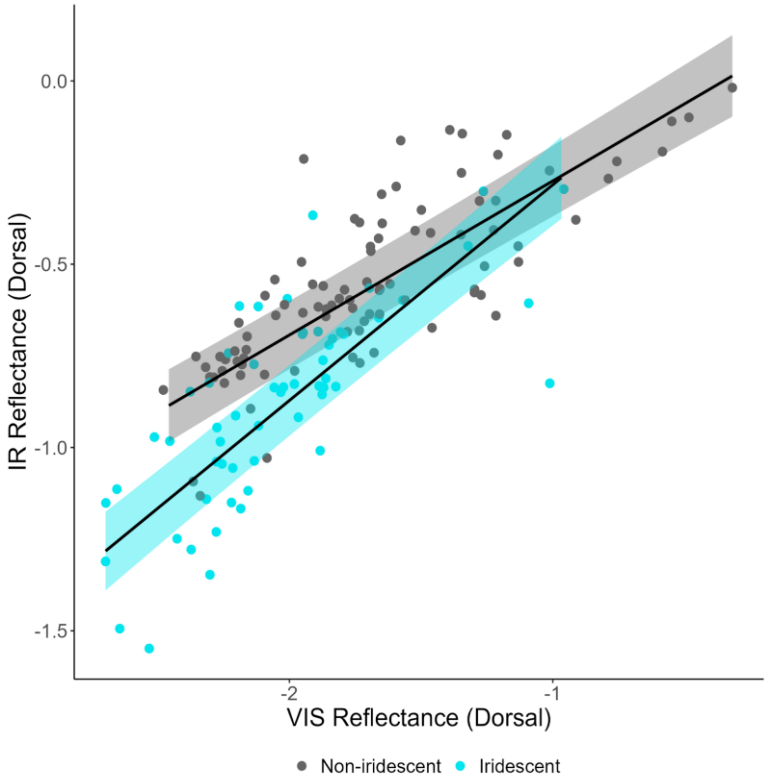


Figure 6: Log VIS reflectance versus log IR reflectance for non-iridescent (grey) and iridescent (blue) species. Points show raw observed data; lines are the predicted conditional effects, (Table 2) and ribbons show the predicted 95% confidence intervals.

Visible and Ultraviolet Reflectance

UV reflectance was not influenced by altitude, stratification, size (Table 3a, b) or dorsal iridescence (Table 3c). Instead, both dorsal and ventral UV reflectance were significantly influenced by VIS reflectance alone (Table 3). Notably, these models are responsible for explaining a low percentage of variance (R^2: <0.04), suggesting a large proportion of UV reflectance is driven by factors outside of the modelled variables.

Table 3: PGLS models predicting dorsal and ventral UV reflectance. Significant predictors or interactions are shown in bold.

a) *Dorsal Ultraviolet Reflectance~ Altitude+Canopy+Size+VIS*

Coefficients	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-0.966	0.517	-1.869	0.0636
Altitude	-0.105	0.252	-0.417	0.677
Canopy	0.5230	0.464	1.142	0.255
Size	-0.044	0.151	-0.294	0.769
VIS	**1.194**	**0.270**	**4.418**	**<0.001**

R^2: 0.115, λ: 0.2

b) *Ventral Ultraviolet Reflectance~ Altitude+Canopy+Size+VIS*

Coefficients	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	0.679	0.421	1.613	0.109
Altitude	-0.028	0.152	-0.184	0.854
Canopy	0.470	0.304	1.546	0.124
Size	0.001	0.112	0.008	0.993
VIS	**2.232**	**0.189**	**11.817**	**0**

R^2: 0.496, λ: 0.554

c) *Dorsal Ultraviolet Reflectance~ altitude + Canopy +Size +VIS+ Iridescence*

Coefficients	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	-0.953	0.506	-1.883	0.062
Altitude	-0.097	0.255	-0.383	0.702
Canopy	0.497	0.465	1.068	0.287
Size	-0.046	0.149	-0.307	0.760
VIS	**1.224**	**0.275**	**4.447**	**<0.001**

Iridescence	0.167	0.270	0.619	0.538
				R^2: 0.11, λ: 0.172

3.5 Discussion:

Our results suggest that the visible reflectance of butterfly wings (i.e., whether wings are darker and melanic or whiter/ more reflective) is shaped more by fine-scale vertical stratification than by broader altitudinal distributions. Whilst we found no evidence that altitude predicted variation in spectral reflectance, we observed that VIS reflectance differed between strata, with canopy species exhibiting overall, darker pigmentation than their understory counterparts. However, stratification explained a small proportion of the observed variation, indicating that additional, unmodelled factors contribute to colour distribution. IR reflectance did not vary with stratification preference independently from VIS reflectance but increases in IR reflectance closely mirrored increases in VIS reflectance, suggesting that visibly darker wings indeed absorb more heat than paler wings. A key result was the role of iridescence in mediating this relationship where, at given levels of VIS reflectance, iridescent species absorbed more IR, indicating that structural colour modulates the passive thermal properties independently of VIS wing reflectance. Reflectance was also significantly impacted by wing size, indicating potential thermoregulatory selection on reflectance at different wing sizes. Finally, VIS and UV reflectance were strongly correlated, yet UV reflectance showed no association with stratification preference, suggesting that UV traits may be maintained by other selective pressures, such as species recognition or sexual signalling.

Reflectance and Environment:

Microclimatic variation within neotropical rainforests is substantial **[16],** and our results corroborate that such environmental niches influence species occurrences (Supplementary Fig. 2). Our results showed no clear differences in wing colouration across altitude, contrary to our predictions and previous studies documenting altitudinal colour shifts across multiple regions, including North America **[32],** Australia **[62]**, Asia **[63]**, Europe **[64],** and Ecuador **[65]**. The absence of an altitudinal pattern in our study may reflect our relatively narrow elevational range (250–1500 masl), compared

with studies spanning higher altitudes, particularly in the context of the Andes (>3000 masl). Alternatively, it may be due to our broad taxonomic sampling, as most previous studies have focussed on intraspecific trends or smaller samples of species, and these trends may not hold up across broader ranges of species. This could be due to contrasting trends being present between different taxa, for example Dufour et al. (2018) **[65]** reported different trends in different genera of butterflies, suggesting that colour - altitude relationships may be lineage-specific.

We did, however, observe a significant difference in VIS colouration across the vertical dimension within the forest. Generally, wings were darker in the canopy, compared to the understory. The thermal conditions between understory and canopy are widely different, with the canopy often reaching higher temperatures than the understory **[23, 14, 15, 52**]. Whilst this initially implies having increased melanin (and therefore more heat absorption) in the canopy is counterintuitive, it is important to acknowledge that the canopy is more variable in temperature and exposed to winds that may affect wing thermoregulation. Whilst we did not look at the difference in dorsal-ventral reflectance across vertical stratification, we cannot rule out the possibility that differences in dorsal and ventral reflectance may further explain potential thermoregulatory mechanisms across strata, as differences in dorsal and ventral reflectance have been shown to vary with thermal environment and may reflect differing requirements for heating and cooling. Furthermore, the thermal function of dorsal and ventral wing surfaces can depend on wing posture, and which surface is exposed to incident solar radiation [36, 28]. Here, where both dorsal and ventral colouration were darker in the canopy, the dorso-ventral differences may be lower than in the understory (Kemp & Krockenberger 2004). This may suggest that the higher dorsoventral contrast in the understory helps maintain flexibility in thermoregulation, allowing butterflies to alter their heat gain and loss depending on which wing surface is exposed to the surrounding environment. In contrast, the more similar dorsal and ventral reflectance in canopy species may reflect selection for more consistent thermal properties across the variable conditions experienced within the canopy.

The exact thermal consequences of melanistic colouration in butterfly thermoregulation also depends on specific behaviours (e.g., basking direction and active thermoregulation) and the environmental

context. For example, some dark insects may orient themselves to minimize direct solar exposure, while others use their dark colour to warm up rapidly for flight **[66, 67**]. As IR reflectance did not vary across stratification once VIS reflectance was controlled for, thermal environment is unlikely to be the key driver of the difference in VIS reflectance across strata. This suggests that the relationship between VIS reflectance and stratification is most likely driven by differences in the light environment and its influence on signalling and camouflage. In the canopy, selection for darker colouration may reflect the combined benefits of visual signalling and camouflage, with dark backgrounds enhancing bright markings used in sexual signalling or aiding predator avoidance **[68-70]**. In contrast, paler colouration in the understory may be driven by signalling needs in low-light conditions, where darker pigmentation, although potentially beneficial for warming, could reduce signal efficacy **[70].** Selection may therefore favour brighter colouration with minimal thermoregulatory cost in the relatively stable understory environment.

For example, the understory species *Morpho helenor* (canopy preference score = 0.01) had light dorsal colouration with high visible reflectance (log VIS reflectance = -1.01). *Morpho* species are known for utilising iridescent colour in directional displays to conspecifics, and flash signalling as a predator deterrent **[84, 85]**. These bright signals may therefore aid signalling in low light conditions through combined wing angles and specific flight behaviours. In contrast, the canopy species *Eunica orphise* (canopy preference score = 0.7), despite also having iridescence, has dark, more melanic wings with reduced visible reflectance (log VIS reflectance = -2.70). *Eunica* butterflies rely on dorsal patterning of iridescent patches, and intricate ventral eyespot patterning in conspecific signalling and mate recognition **[86]**. Here, the dark contrast of the wing against these patterns are likely to enhance the dorsal pattern signal whilst having a potential thermoregulatory benefit through decreased infrared to maintain temperatures in a more variable, windy canopy environment.

Reflectance and Size

Butterfly spectral responses in the VIS and IR were significantly affected by wing size. Our results revealed that larger butterflies were darker in the VIS and IR than small butterflies. Notably, on the dorsal wing surface, this relationship was stronger in the IR compared to the VIS reflectance,

suggesting the difference in reflectance across sizes may be driven by a thermoregulatory effect. The manoeuvrability and aerodynamics of butterflies is complex and determined by a range of factors including wing shape, wing shape to size ratios and body mass **[71,72].** There are multiple trade-offs associated with increases in wing and body size. Larger butterflies require more energy for flight, benefiting from a smaller surface area to volume ratio, allowing them to maintain heat more efficiently, but heat up more slowly **[73-77]**. Selection may favour darker melanistic colouration in larger species enabling them to heat up faster for flight. Smaller butterflies are less likely to experience this challenge, as they require less energy needed to fly, and their greater surface area to volume ratio allows increased heat exchange with the environment. Importantly, these results suggest that the thermoregulatory byproducts of wing reflectance are significantly impacted by wing size, which may impact habitat distributions and aerodynamics of tropical butterflies.

Visible Reflectance, Infrared Reflectance and Iridescence

Based on the thermal melanism hypothesis, melanic individuals generally absorb more solar radiation and heat up faster than pale individuals **[5]**. We found that increases in IR reflection closely tracked increases in the VIS, evidencing that visibly darker wings do indeed absorb more heat than paler wings. Live butterflies commonly use a combination of active mechanisms to regulate their wing temperature **[27]**. Here, we found that even without these active thermoregulatory mechanisms, the reflection and absorption of IR is maintained in dead wings. This suggests wing pigmentation has a subsequent effect on passive heat exchange. However, we found that the relationship between VIS and IR is significantly mediated by iridescence. At a given VIS reflectance, iridescence allowed species to achieve higher absorbance of IR light, implying that the presence of structural colour results in lower absolute IR reflectance. Whilst the presence or absence of iridescence was not significantly impacted by environmental factors, structural colour gives potential for increased decoupling of IR and VIS reflectance, allowing these to evolve separately. Together these results confirm our hypotheses that structural and pigmentary properties of the wing contribute to passive thermoregulation.

Butterfly wing nanostructures that generate VIS colour also influence thermal properties by mediating heat absorption, reflection, and dissipation. Previous work shows that iridescent scales can either enhance IR reflectance to prevent overheating or increase absorption to facilitate warming, often via thickened chitin layers that aid IR dissipation. Chitin inherently has the capacity to absorb and emit heat, and thickening of chitin layers within the nanostructure can increase the emissivity of heat from these areas **[27, 45, 78-80]**. Our results support this, as iridescent species exhibited significantly lower IR reflectance than non-iridescent species, indicating greater IR absorption. Many iridescent thin-film nanostructures also incorporate melanin, which enhances VIS colouration, as shown in *Morpho* butterflies **[81-83].** Consistent with this, iridescent species were darker in the VIS compared to non-iridescent species, implying the structural colour is often paired with melanised scales. This has further implications for both VIS and IR optical properties of wings, as colour enhancement of iridescent signals may result in a secondary thermoregulatory effect. We found that melanin-rich, colour-producing nanostructures absorbed more radiation across both VIS and IR wavelengths. Because melanin strongly absorbs IR radiation, the combination of melanisation and scale nanostructure likely drives the reduced IR reflectance observed in iridescent butterflies. Although the IR properties of melanised thin-film multilayers remain poorly studied **[46],** the correlation between VIS and IR reflectance is likely to lead to evolutionary trade-offs between visual signalling and thermoregulation. Selection for iridescence may therefore simultaneously affect signalling efficiency and thermal balance, with fitness consequences across different thermal environments.

Phylogeny and Constraint

As expected, **[87-89],** our results showed butterfly colour had a moderate to high phylogenetic signal, indicating that closely related species tended to share similar optical traits. Despite this strong phylogenetic structuring, we still detected significant effects of environmental variables on coloration. This pattern suggests that, while phylogeny is an important correlate of colour traits, environmental factors additionally shape variation in optical properties across species, within the constraints of individual evolutionary history.

Reflective properties were broadly conserved between dorsal and ventral wing surfaces, with pale dorsal wings corresponding to pale ventral wings, and vice versa. The strength of this dorsoventral correlation varied between spectra, being strongest in the IR, suggesting thermoregulatory properties of both wing surfaces are maintained consistently. The relationship was weaker in the VIS and weakest in the UV, where dorsal and ventral wings, despite not being optically isolated, serve different signalling roles **[90, 91]**, allowing greater scope for independent selection on dorso–ventral signalling functions **[92]**.Here, we observed a strong positive relationship between VIS and UV reflectance, where darker individuals reflected less UV, and paler individuals reflected more. Melanin absorbs and dissipates harmful UV radiation **[40-42**], consistent with our finding that darker individuals exhibited reduced UV reflectance. However, variation in UV reflectance was generally poorly explained by the variables in our models, suggesting that its evolution is relatively unconstrained by the environment and correlations with other parts of the spectrum, particularly IR, so it may be able to evolve more rapidly in response to other selective pressures such as species recognition or sexual mate signalling.

Conclusions

This study contributes to a more comprehensive understanding of animal colouration and the interplay of optical properties in tropical butterflies. We found evidence that vertical microclimates within forests shape wing pigmentation of tropical butterflies, while there was no evidence for an effect of altitude across the sites we studied, suggesting that signalling and camouflage are key drivers of colouration in canopy and understory species. Although reflectance in spectral categories was generally correlated, these appear to subtly respond differently to different selective pressures such as size and stratification. One way the de-coupling across spectra could be achieved is through structural colour. This study's findings suggest pigmentary and structural colouration influence passive thermoregulatory properties, providing grounds for further research on the explicit roles of nanostructure and pigmentation in passive thermoregulation. Finally, our results emphasise the multifunctional nature of butterfly colouration, suggesting that thermal, ecological, and signalling pressures alongside size and dorsal-ventral differences interact to maintain the diversity of optical traits found across tropical butterfly communities.

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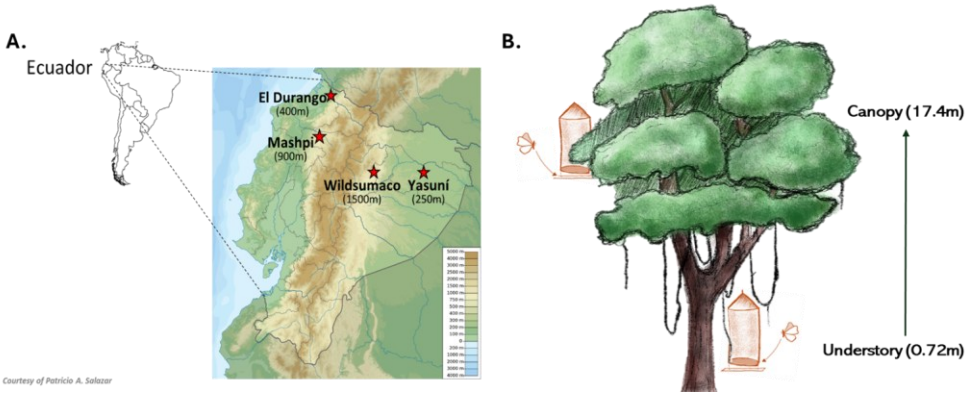
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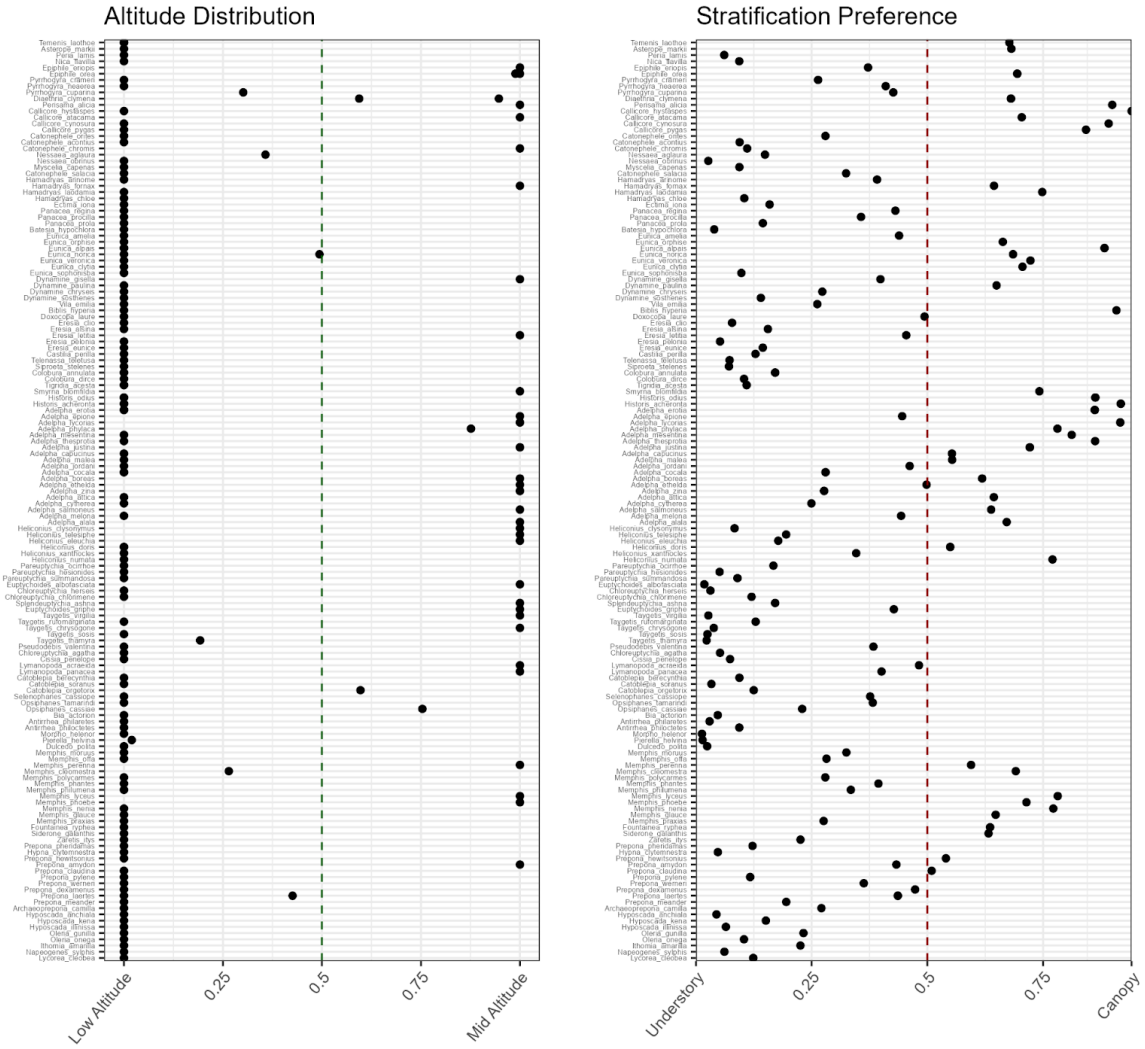
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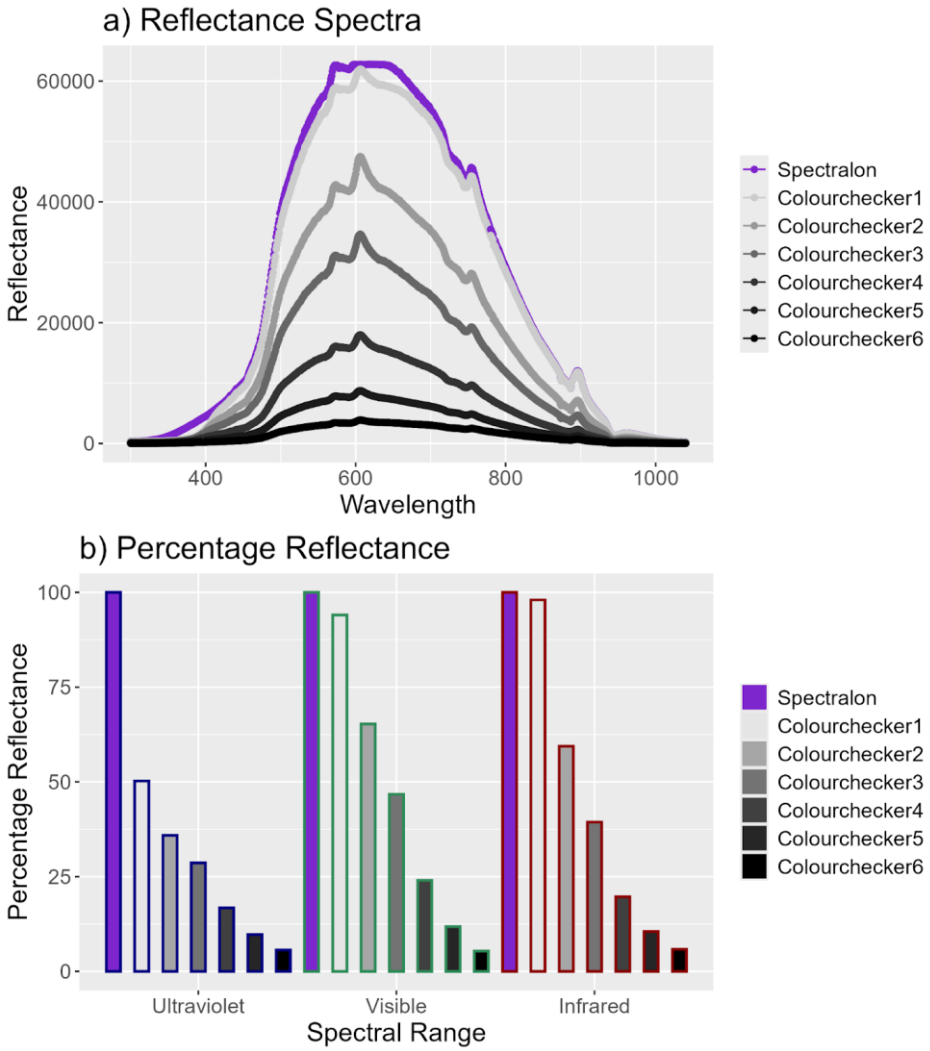
3.7 Supplementary Figures



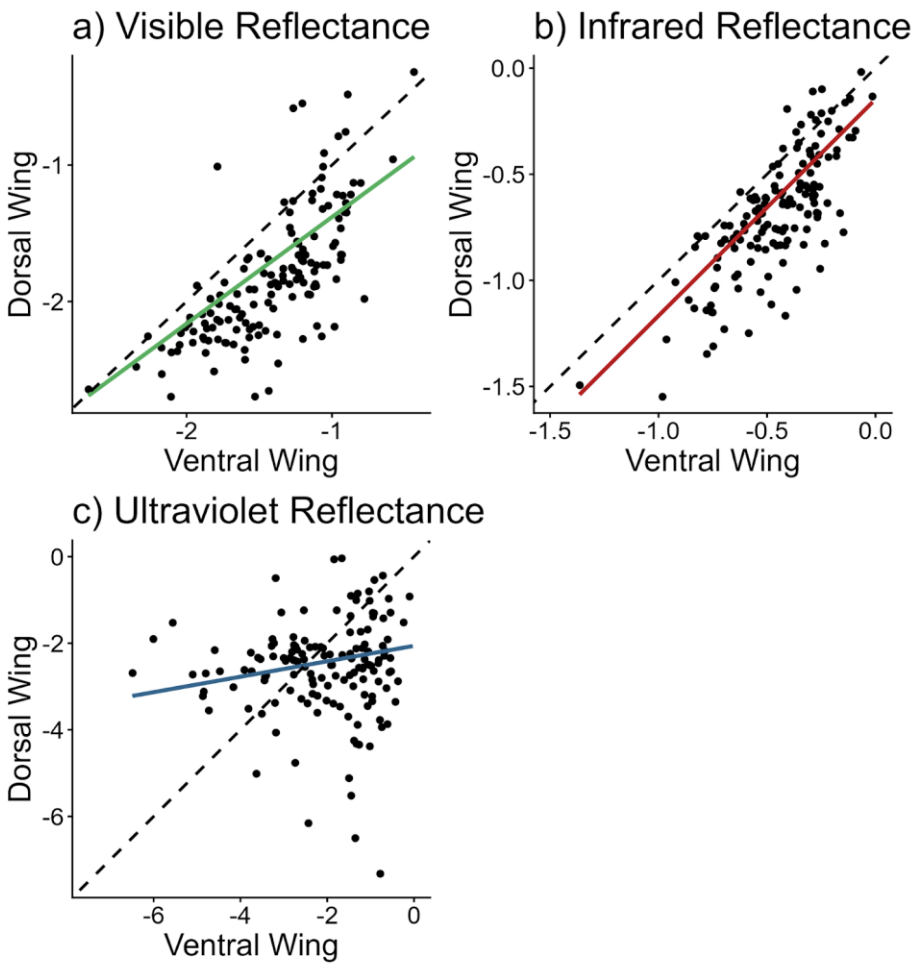
Supplementary Figure 1: A: The four trapping sites in Ecuador: Yasuni National Park, 250m; Wildsumaco Lodge, at 1500m; Mashpi Rainforest Reserve, 900m and El Durango, 400m, courtesy Patricio A. Salazar. B: An illustration of the trapping positions for each trap pair in the understory and the canopy, and the median distance from the ground (in meters).



Supplementary Figure 2: The altitude distribution and preference for Understory and canopy for each sampled species. The dotted lines indicate where species were found equally in both habitats.



Supplementary Figure 3: (a) The reflectance of the grayscale colour checker standard from 300nm-1200nm, compared to the reference standard of a spectralon standard coloured in purple. (b) The calculated percentage reflectance from the greyscale colour standards, used to calibrate images in the UV, VIS and IR ranges.



Supplementary Figure 4: Dorsal and ventral wing reflectance (log-transformed) across spectra. (a) visible, (b) infrared, and (c) ultraviolet reflectance. Values are log-transformed RGB pixel measurements for each species. The dotted line indicates a 1:1 ratio.

3.8 Supplementary Tables:

Supplementary Table 1: Table of Species

Subfamily	Tribe	Genus	Species	Altitude	Stratification	Side of Andes	Taxonomic Reference
Danainae	Danaini	Lycorea	cleobea	Low	Understory	East	E. Doubleday, [1847]
Danainae	Ithomiini	Napeogenes	sylphis	Low	Understory	East	Guérin-Méneville, [1844]
Danainae	Ithomiini	Ithomia	amarilla	Low	Understory	East	Haensch, 1903
Danainae	Ithomiini	Hyposcada	illinissa	Low	Understory	East	Hewitson, [1852]
Danainae	Ithomiini	Hyposcada	kena	Low	Understory	East	Hewitson, 1872
Danainae	Ithomiini	Hyposcada	anchiala	Low	Understory	East	Hewitson, 1868
Danainae	Ithomiini	Oleria	onega	Low	Understory	East	Hewitson, [1852])
Danainae	Ithomiini	Oleria	gunilla	Low	Understory	East	Hewitson, 1858
Heliconiinae	Heliconiini	Heliconius	xanthocles	Low	Understory	East	H. Bates, 1862
Heliconiinae	Heliconiini	Heliconius	doris	Low	Canopy	Both	Linnaeus, [1771]
Heliconiinae	Heliconiini	Heliconius	numata	Low	Canopy	East	Cramer, 1780
Heliconiinae	Heliconiini	Heliconius	eleuchia	Mid	Understory	West	Hewitson, [1854]
Heliconiinae	Heliconiini	Heliconius	telesiphe	Mid	Understory	East	E. Doubleday, 1847
Heliconiinae	Heliconiini	Heliconius	clysonymus	Mid	Understory	East	Latreille, [1817]
Limenitidinae	Limenitidini	Adelpha	alala	Mid	Canopy	East	Hewitson, 1847
Limenitidinae	Limenitidini	Adelpha	melona	Low	Understory	East	Hewitson, 1847
Limenitidinae	Limenitidini	Adelpha	salmoneus	Mid	Canopy	East	A. Butler, 1866
Limenitidinae	Limenitidini	Adelpha	cytherea	Low	Understory	East	Linnaeus, 1758
Limenitidinae	Limenitidini	Adelpha	attica	Low	Canopy	East	C. Felder & R. Felder, 1867

Limenitidinae	Limenitidini	Adelpha	zina	Mid	Understory	Both	Hewitson, 1867
Limenitidinae	Limenitidini	Adelpha	ethelda	Mid	Understory	West	Hewitson, 1867
Limenitidinae	Limenitidini	Adelpha	boreas	Mid	Canopy	East	A. Butler, 1866
Limenitidinae	Limenitidini	Adelpha	cocala	Low	Understory	Both	Cramer, 1779
Limenitidinae	Limenitidini	Adelpha	jordani	Low	Understory	East	Fruhstorfer, 1913
Limenitidinae	Limenitidini	Adelpha	malea	Low	Canopy	East	C. Felder & R. Felder, 1861
Limenitidinae	Limenitidini	Adelpha	capucinus	Low	Canopy	East	Walch, 1775
Limenitidinae	Limenitidini	Adelpha	justina	Mid	Canopy	Both	C. Felder & R. Felder, 1861
Limenitidinae	Limenitidini	Adelpha	thesprotia	Low	Canopy	East	C. Felder & R. Felder, 1867
Limenitidinae	Limenitidini	Adelpha	mesentina	Low	Canopy	East	Cramer, 1777
Limenitidinae	Limenitidini	Adelpha	phylaca	Mid	Canopy	West	H. Bates, 1866
Limenitidinae	Limenitidini	Adelpha	lycorias	Mid	Canopy	Both	Godart, [1824]
Limenitidinae	Limenitidini	Adelpha	epione	Mid	Understory	East	Godart, [1824]
Limenitidinae	Limenitidini	Adelpha	erotia	Low	Canopy	Both	Hewitson, 1847
Apaturinae	Apaturinae	Doxocopa	laure	Low	Understory	East	Drury, 1773
Biblidinae	Catonephelini	Eunica	sophonisba	Low	Understory	East	Cramer, 1780
Biblidinae	Catonephelini	Eunica	veronica	Low	Canopy	East	H. Bates, 1864
Biblidinae	Eubagini	Dynamine	sosthenes	Low	Understory	East	Hewitson, 1869
Biblidinae	Epiphilini	Pyrrhogyra	cuparina	Low	Understory	East	H. Bates, 1865
Biblidinae	Catonephelini	Eunica	orphise	Low	Canopy	East	Cramer, 1775
Biblidinae	Catonephelini	Eunica	amelia	Low	Understory	East	Cramer, 1777
Biblidinae	Catonephelini	Eunica	alpais	Low	Canopy	Both	Godart, [1824]

Biblidinae	Ageroniini	Hamadryas	fornax	Mid	Canopy	East	Hübner, [1823]
Biblidinae	Biblidini	Vila	emilia	Low	Understory	East	Cramer, 1779
Biblidinae	Ageroniini	Hamadryas	arinome	Low	Understory	East	Lucas, 1853
Biblidinae	Ageroniini	Hamadryas	chloe	Low	Understory	East	Stoll, 1787
Biblidinae	Ageroniini	Ectima	iona	Low	Understory	East	E. Doubleday, [1848]
Biblidinae	Ageroniini	Batesia	hypochlora	Low	Understory	East	C. Felder & R. Felder, 1862
Biblidinae	Eubagini	Dynamine	chryseis	Low	Understory	East	H. Bates, 1865
Biblidinae	Ageroniini	Panacea	procilla	Low	Understory	East	Hewitson, [1854]
Biblidinae	Ageroniini	Panacea	regina	Low	Understory	East	H. Bates, 1864
Biblidinae	Ageroniini	Panacea	prola	Low	Understory	East	E. Doubleday, [1848]
Biblidinae	Callicorini	Asterope	markii	Low	Canopy	East	Hewitson, 1857
Biblidinae	Callicorini	Callicore	atacama	Mid	Canopy	West	Hewitson, 1852
Biblidinae	Callicorini	Perisama	alicia	Mid	Canopy	East	Hewitson, 1868
Biblidinae	Ageroniini	Hamadryas	laodamia	Low	Canopy	West	Cramer, 1777
Biblidinae	Callicorini	Callicore	hystaspes	Low	Canopy	East	Drury, 1782
Biblidinae	Callicorini	Diaethria	clymena	Mid	Canopy	Both	Cramer, 1775
Biblidinae	Epiphilini	Nica	flavilla	Low	Understory	East	Godart, [1824]
Biblidinae	Biblidini	Biblis	hyperia	Low	Canopy	East	Cramer, 1779
Biblidinae	Catonephelini	Eunica	norica	Low	Canopy	Both	Hewitson, 1852
Biblidinae	Epiphilini	Epiphile	orea	Mid	Canopy	Both	Hübner, [1823]
Biblidinae	Epiphilini	Epiphile	eriopis	Mid	Understory	West	Hewitson, 1857
Biblidinae	Epiphilini	Pyrrhogyra	neaerea	Low	Understory	East	Linnaeus, 1758

Biblidinae	Epiphilini	Pyrrhogyra	crameri	Low	Understory	East	Aurivillius, 1882
Biblidinae	Epiphilini	Peria	lamis	Low	Understory	East	Cramer, 1779
Biblidinae	Catonephelini	Catonephele	salacia	Low	Understory	East	Hewitson, 1852
Biblidinae	Catonephelini	Myscelia	capenas	Low	Understory	East	Hewitson, [1857]
Biblidinae	Catonephelini	Catonephele	acontius	Low	Understory	East	Linnaeus, 1771
Biblidinae	Catonephelini	Catonephele	orites	Low	Understory	West	Stichel, 1899
Biblidinae	Catonephelini	Catonephele	chromis	Mid	Understory	East	E. Doubleday, [1848]
Biblidinae	Catonephelini	Nessaea	obrinus	Low	Understory	East	Linnaeus, 1758
Biblidinae	Catonephelini	Nessaea	aglaura	Low	Understory	West	E. Doubleday, [1848]
Biblidinae	Callicorini	Callicore	pygas	Low	Canopy	East	Godart, [1824]
Biblidinae	Eubagini	Dynamine	paulina	Low	Canopy	East	H. Bates, 1865
Biblidinae	Eubagini	Dynamine	gisella	Mid	Understory	East	Hewitson, 1857
Biblidinae	Epiphilini	Temenis	laothoe	Low	Canopy	Both	Cramer, 1777
Biblidinae	Callicorini	Callicore	cynosura	Low	Canopy	East	E. Doubleday, [1847]
Biblidinae	Catonephelini	Eunica	clytia	Low	Canopy	East	Hewitson, 1852
Nymphalinae	Coeini	Historis	acheronta	Low	Canopy	Both	Fabricius, 1775
Nymphalinae	Coeini	Historis	odius	Low	Canopy	Both	Fabricius, 1775
Nymphalinae	Nymphalini	Smyrna	blomfildia	Mid	Canopy	West	Fabricius, 1781
Nymphalinae	Nymphalini	Tigridia	acesta	Low	Understory	Both	Linnaeus, 1758
Nymphalinae	Nymphalini	Colobura	dirce	Low	Understory	East	Linnaeus, 1758
Nymphalinae	Nymphalini	Colobura	annulata	Low	Understory	Both	Willmott, Constantino & J. Hall, 2001
Nymphalinae	Victorinini	Siproeta	stelenes	Low	Understory	East	Linnaeus, 1758

Nymphalinae	Melitaeini	Telenassa	teletusa	Low	Understory	East	Godart, [1824]
Nymphalinae	Melitaeini	Castilia	perilla	Low	Understory	East	Hewitson, 1852
Nymphalinae	Melitaeini	Eresia	eunice	Low	Understory	East	Hübner, [1807]
Nymphalinae	Melitaeini	Eresia	letitia	Mid	Understory	East	Hewitson, 1869
Nymphalinae	Melitaeini	Eresia	alsina	Low	Understory	West	Hewitson, 1869
Nymphalinae	Melitaeini	Eresia	clio	Low	Understory	West	Linnaeus, 1758
Nymphalinae	Melitaeini	Eresia	pelonia	Low	Understory	East	Hewitson, 1852
Charaxinae	Anaeini	Fountainea	ryphea	Low	Canopy	West	Cramer, 1775
Charaxinae	Anaeini	Memphis	polycarmes	Low	Understory	East	Fabricius, 1775
Charaxinae	Anaeini	Memphis	cleomestra	Low	Canopy	West	Hewitson, 1869
Charaxinae	Anaeini	Memphis	offa	Low	Understory	East	H. Druce, 1877
Charaxinae	Anaeini	Memphis	moruus	Low	Understory	East	Fabricius, 1775
Charaxinae	Anaeini	Memphis	perenna	Mid	Canopy	West	Godman & Salvin, 1884
Charaxinae	Anaeini	Memphis	philumena	Low	Understory	East	E. Doubleday, [1849]
Charaxinae	Anaeini	Memphis	phantes	Low	Understory	East	Hopffer, 1874
Charaxinae	Anaeini	Memphis	phoebe	Mid	Canopy	East	Hopffer, 1874
Charaxinae	Anaeini	Memphis	lyceus	Mid	Canopy	West	Hopffer, 1874
Charaxinae	Anaeini	Memphis	nenia	Low	Canopy	West	H. Druce, 1877
Charaxinae	Anaeini	Memphis	praxias	Low	Understory	East	Hopffer, 1874
Charaxinae	Anaeini	Memphis	glauce	Low	Canopy	West	C. Felder & R. Felder, 1862
Charaxinae	Anaeini	Zaretis	itys	Low	Understory	East	Cramer, 1777
Charaxinae	Anaeini	Siderone	galanthis	Low	Canopy	East	Cramer, 1775

Charaxinae	Anaeini	Hypna	clytemnestra	Low	Understory	West	Cramer, 1777
Charaxinae	Preponini	Prepona	pheridamas	Low	Understory	East	Cramer, 1777
Charaxinae	Preponini	Archaeoprepona	camilla	Low	Understory	West	Godman & Salvin, 1884
Charaxinae	Preponini	Prepona	meander	Low	Understory	East	Cramer, 1775
Charaxinae	Preponini	Prepona	dexamenus	Low	Understory	East	Hopffer, 1874
Charaxinae	Preponini	Prepona	werneri	Low	Understory	West	M. Hering & Hopp, 1925
Charaxinae	Preponini	Prepona	pylene	Low	Understory	East	Hewitson, [1854]
Charaxinae	Preponini	Prepona	amydon	Mid	Understory	West	Hewitson, [1854])
Charaxinae	Preponini	Prepona	hewitsonius	Low	Canopy	East	H. Bates, 1860
Charaxinae	Preponini	Prepona	claudina	Low	Canopy	East	Godart, [1824]
Charaxinae	Preponini	Prepona	laertes	Low	Understory	Both	Hübner, [1811]
Satyrinae	Morphini	Antirrhea	philoctetes	Low	Understory	East	Linnaeus, 1758
Satyrinae	Morphini	Antirrhea	philaretes	Low	Understory	West	C. Felder & R. Felder, 1862
Satyrinae	Morphini	Morpho	helenor	Low	Understory	East	Cramer, 1776
Satyrinae	Brassolini	Selenophanes	cassiope	Low	Understory	East	Cramer, 1775
Satyrinae	Brassolini	Catoblepia	orgetorix	Mid	Understory	West	Hewitson, 1870
Satyrinae	Brassolini	Catoblepia	soranus	Low	Understory	East	Westwood, 1851
Satyrinae	Brassolini	Catoblepia	berecynthia	Low	Understory	East	Cramer, 1777
Satyrinae	Brassolini	Opsiphanes	cassiae	Mid	Understory	East	Linnaeus, 1758
Satyrinae	Brassolini	Opsiphanes	tamarindi	Low	Understory	West	C. Felder & R. Felder, 1861
Satyrinae	Brassolini	Bia	actorion	Low	Understory	East	C. Felder & R. Felder, 1861
Satyrinae	Haeterini	Dulcedo	polita	Low	Understory	West	Hewitson, 1869

Satyrinae	Haeterini	Pierella	helvina	Low	Understory	West	Hewitson, 1859
Satyrinae	Satyrini	Chloreuptychia	chlorimene	Low	Understory	East	Hübner, [1819]
Satyrinae	Satyrini	Chloreuptychia	herseis	Low	Understory	East	Godart, [1824]
Satyrinae	Satyrini	Euptychoides	albofasciata	Mid	Understory	East	Hewitson, 1869
Satyrinae	Satyrini	Pareuptychia	summandosa	Low	Understory	East	Gosse, 1880
Satyrinae	Satyrini	Pareuptychia	hesionides	Low	Understory	East	Forster, 1964
Satyrinae	Satyrini	Pareuptychia	ocirrhoe	Low	Understory	West	Fabricius, 1776
Satyrinae	Satyrini	Euptychoides	griphe	Mid	Understory	East	C. Felder & R. Felder, 1867
Satyrinae	Satyrini	Splendeuptychia	ashna	Mid	Understory	East	Hewitson, 1869
Satyrinae	Satyrini	Taygetis	thamyra	Low	Understory	East	Cramer, 1779
Satyrinae	Satyrini	Taygetis	sosis	Low	Understory	East	Hopffer, 1874
Satyrinae	Satyrini	Taygetis	chrysogone	Mid	Understory	East	E. Doubleday, 1849
Satyrinae	Satyrini	Taygetis	rufomarginata	Low	Understory	West	Staudinger, 1888
Satyrinae	Satyrini	Taygetis	virgilia	Mid	Understory	East	Cramer, 1776
Satyrinae	Satyrini	Pseudodebis	valentina	Low	Understory	East	Cramer, 1779
Satyrinae	Satyrini	Chloreuptychia	agatha	Low	Understory	East	A. Butler, 1867
Satyrinae	Satyrini	Cissia	penelope	Low	Understory	East	Fabricius, 1775
Satyrinae	Satyrini	Lymanopoda	panacea	Mid	Understory	East	Hewitson, 1869
Satyrinae	Satyrini	Lymanopoda	acraeida	Mid	Understory	East	A. Butler, 1868

Supplementary Table 2: A table of the phylogenetic signal (Pagels lambda) for the response and predictor variables used in subsequent modelling.

Variable	**Pagel's λ**	***Lower Likelihood***	***Upper Likelihood***
Canopy	0.851	0.673	0.942
Size	0.990	0.956	1
Visible Dorsal	0.981	0.908	1
Visible Ventral	0.770	0.443	0.925
IR Dorsal	0.775	0.507	0.935
IR Ventral	0.650	0.307	0.880
UV Dorsal	0.967	0.890	0.993
UV Ventral	0.835	0.473	0.949

Supplementary Table 3: PGLS model coefficients predicting ventral infrared reflectance. Significant predictors are shown in bold.

Ventral Infrared Reflectance~ VIS+Canopy+Size

Coefficients	**Estimate**	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	0.220	0.066	3.331	0.001
VIS	**0.476**	**0.0241**	**19.777**	**0**
Canopy	0.002	0.040	0.038	0.970
Size	-0.009	0.016	-0.582	0.561

4.0 Altitude and canopy-cover shape passive heat gain and active thermoregulation in tropical butterflies

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Data Availability: The data is available from https://figshare.com/s/ad52e8bfdbccf00425b6

Diversity and Inclusion Statement: Our study is the result of an equitable international collaboration involving researchers based in both the UK and Ecuador, representing a range of career stages from research assistants to principal investigators. Authors collaboratively contributed diverse perspectives which were considered throughout the entire project. Where relevant, literature published in both English and Spanish was revised and cited. We are committed to open and accessible science, and all data, code, and supplementary materials associated with this study will be made openly available.

Keywords: Altitude; Butterfly Temperature; Elevation; Solar Irradiance; Thermoregulation; Tropical Butterflies

Title

Altitude and canopy-cover shape passive heat gain and active thermoregulation in tropical butterflies

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Keywords: Altitude; Butterfly Temperature; Elevation; Solar Irradiance; Thermoregulation; Tropical Butterflies

4.1 Abstract

Across altitudinal gradients, there are distinct thermal environments, driven by changes in solar radiation and air temperature, where high elevations have cooler air temperatures but receive greater solar radiation, while lowlands have warmer air temperatures but reduced solar radiation. In ectothermic butterflies, who often gain heat via basking, the impact of altitude and air temperatures on thermoregulation is not well understood, particularly for tropical species. We investigated how mounted *Heliconius erato* and *Heliconius sara* wings and live butterfly body temperatures respond to variation in altitude, ambient temperature, and forest cover, distinguishing between passive and active thermoregulatory mechanisms. Passive wing heating was strongly mediated by forest cover and altitude. In open conditions, wings heated faster than under canopy cover, with this effect amplified at higher altitudes. As a result, wings at higher altitude were warmer relative to air temperature when exposed to direct sunlight, likely due to increased solar radiation. In contrast, thoracic temperatures of live butterflies showed a different pattern. Individuals at higher altitudes tended to be cooler relative to ambient temperature, although this difference diminished at lower air temperatures. Thoracic temperatures consistently exceeded ambient conditions more than in wings, indicating that heat gain is not solely passive and possibly involves metabolic heat production from thoracic muscles. Together, these findings show that tropical butterflies employ a combination of physiological and behavioural thermoregulation to maintain functional body temperatures across altitudes and microclimates. These complex thermoregulatory strategies may allow greater flexibility under changing climatic conditions in tropical forests and highlight the importance of considering both air temperature and solar exposure in ectotherm responses.

Summary Statement: Butterflies regulate body temperature differently from isolated wings across altitudes, highlighting the roles of solar radiation and active heat production in thermal balance.

4.2 Introduction

Climate change is increasingly shaping how animals adapt to variable environmental conditions, particularly in habitats that are rapidly changing (Bonan 2008; Feeley et al., 2017; Sheldon, 2019). In tropical forest systems, both atmospheric changes and structural shifts in canopy cover alter the environmental conditions that species occupy. While neotropical rainforests typically exhibit low seasonality (Sheldon, 2019; Montejo-Kovacevich et al., 2020; Blumthaler et al 1997), they are characterized by strong spatial heterogeneity. The complexity in the environmental structure of tropical forests creates ecological niches across vertical and horizontal gradients.

Altitudinal variation between neotropical forests is associated with large changes in temperature, solar radiation, humidity, precipitation, winds and plant composition, all of which strongly influence the distribution and physiology of forest insects, such as butterflies (Kovacevic et al., 2019; Abram et al., 2017; Aparecido et al., 2018; Sheldon, 2019; Royer et al., 2011; Blumthaler et al., 1997). Increases in solar radiation with altitude depend on an array of factors from the specific region to the cloudiness and season (Blumthaler et al., 1997; Spence & Tingley., 2020; Kumar et al., 1997; Buckley et al., 2013). Existing studies on solar exposure across tropical altitudinal gradients have found notable changes in solar radiation across an altitudinal gradient of up to 1000m, where increases in altitude are associated with increases in solar radiation (Janzen, 1967; Sola et al., 2008; Fyllas et al., 2017; Hoyos-Gomez & Ruiz-Mendoza 2021). This shows that even small shifts in altitude give rise to differences in solar radiation and thermal environments, creating substantial differences in environments across tropical altitude gradients.

As ectotherms, butterflies are especially sensitive to environmental temperatures, and their ability to thermoregulate is critical for survival and activity in variable tropical habitats (Kovacevic et al., 2019). Thermoregulatory strategies in butterflies involve a combination of behavioural and

physiological mechanisms that allow the regulation of both wing and body temperature. Wing and body thermoregulation differ in both function and sensitivity to heat (Kingsolver & Woods, 2016; Kingsolver & Koehl, 1985; Tsai et al., 2020). The thorax, where the flight muscles are housed, is typically maintained at more stable temperatures via physiological processes, including the contraction of flight muscles and the movement of haemolymph through the body. This haemolymph flow also extends into the wings, where it enhances convective heat exchange, though wings are generally more thermally labile than the thorax (Clark et al., 1973; Kingsolver, 1988; Kingsolver & Koehl, 1985; Tsai et al., 2020). The temperature of wings is regulated separately from the body, and butterflies utilise radiative cooling and heating from the wing surface through passive and behavioural thermoregulation, such as basking, wing positioning, or habitat selection. This further enables butterflies to manipulate the absorption and reflection of solar radiation (i.e., heat) from the wings (Tsai et al., 2020; Kingsolver & Moffat, 1982; Clench, 1966; Kingsolver & Koehl, 1985). These mechanisms become increasingly important in cooler or more variable climates, such as those found at higher elevations, to maintain temperatures, and in warmer climates to avoid overheating.

Given that solar radiation and ambient temperature change with elevation, we expect butterfly thermoregulation to vary across altitudinal gradients. Thermal conformity, as described by Angilletta (2009), predicts that ectothermic body temperatures track ambient temperatures closely, whereas thermal regulators may deviate from ambient temperatures through active or passive mechanisms. The level of thermal conformity in butterflies therefore may shift between altitudes. Although ambient temperature generally decreases with altitude, levels of solar radiation tend to increase with altitude due to reduced atmospheric filtering (Körner, 2007; Royer et al., 2011; Iqbal, 2012; Vignola et al., 2019). Alongside shifts in altitude, canopy cover, and light exposure is also likely to impact butterfly thermodynamics. Shading from the forest canopy affects how radiation reaches the butterfly and how effectively heat can be absorbed or retained (Royer et al., 2011; Iqbal, 2012; Becker et al., 1989; Pearcy et al., 1990; Breshears et al., 1997). Thus, the level of solar radiation alongside ambient temperature is likely to impact thermoregulation in butterflies.

According to Newton's Law of Cooling (Winterton, 1999), the rate of heat exchange depends on the temperature gradient between the body and the surrounding environment, such that body temperature should tend towards air temperature. This is further driven by convective properties of the body surface, where active hemolymph flow determines the rate of heat transfer between the body and surrounding air. Whilst live wings utilise convective heat transfer, there are additional passive heat transfer mechanisms that drive heat exchange. Without convection, passive heat exchange in wings may result in an additional effect of solar radiation. Therefore, where ambient temperatures are lower but solar radiation is higher, wings may reach high temperatures through increased absorption, particularly in open-canopy conditions. Insights into how thermoregulation in tropical butterflies is affected by the conflicting effects of air temperature and solar radiation at different altitudes is important for understanding how they will respond to changing environmental temperatures (Bonebrake et al., 2014).

Passive heating from solar radiation plays a role in surface warming for ectotherms, especially in butterfly wings, which lack the same thermoregulatory control as the thorax (Liao et al., 2017). Differences in wing coloration can influence the thermoregulatory properties of the wing. Butterfly wings contain a variety of pigments and nanostructures that interact with light across the visible, ultraviolet and infrared spectra (Ingram & Parker, 2008; Clusella-Trullas et al., 2008; Lloyd & Nadeau, 2021). Melanin, for example, efficiently absorbs across a broad spectrum and is responsible for converting ultraviolet wavelengths into heat. This is consistent with the Stefan–Boltzmann law (Tiihonen, 1997; Incropera & DeWitt, 1996), which predicts that darker and more absorptive surfaces will radiate and absorb energy more effectively, especially under direct solar exposure (Incropera & DeWitt, 1996). We therefore expect melanised regions of the wing to warm more quickly than other wing regions (Kayser, 2013; Stavenga et al., 2014; Clusella-Trullas et al., 2008; Kettlewell, 1973). Red ommochromes and yellow 3OHK pigments found in butterfly wings also interact with visible light, however their infrared optical properties are less known (Kayser, 2013; Stavenga et al., 2014; Kingsolver, 1988).

The neotropical species *Heliconius erato* and *Heliconius sara* span an altitudinal range from sea level to just over 1,000 metres above sea level and experience a stable range of temperatures at either end of this (Montejo-Kovacevich 2020). Moreover, they display both pigmentary and structural colour differences and are closely related. Both species have melanised black regions and 3OHK yellow patches (Wilts et al., 2017; Bybee et al., 2012). Additionally, *H. erato* has red ommochrome pigmentary colour, whereas *H. sara* exhibits blue iridescence with melanin-rich nanostructures (Parnell et al. 2017), which may enhance heat absorption and emission (Krishna et al 2020). We predict that wing temperatures of *H. erato* and *H. sara* will increase with ambient temperature, but that the degree of heating will depend on altitude, canopy openness, and wing colour.

To disentangle the effects of the environment on passive and active thermoregulation, we measured the temperatures of *H. sara*, and *H. erato* wings and bodies in the field. We include measurements of live butterfly bodies and dead wing specimens, the latter acting as passive heating controls without physiological or behavioural thermoregulation. We expect dead wings to conform closely to air temperature and be influenced by solar radiation, indicating passive heating dynamics. Conversely, live butterflies may show greater deviation from ambient air due to thermoregulation by physiological mechanisms, such as haemolymph flow and heat generation, or behavioural mechanisms such as basking or shade seeking. We also expect that butterfly dead wing and live body temperatures distinctly vary with altitude and canopy cover. By comparing dead wing and live body temperatures across altitudes, cover types, and species, we aim to understand how tropical butterflies modulate their temperature under different environmental conditions.

4.3 Materials and Methods

Wing Temperature in butterfly models: Experimental Design and Data Collection

Wing temperature experiments were conducted at two sites during the wet season in east Ecuador across 9 days (30th July – 7th August 2024). The selected sites were at low (390m) and mid (1500m) altitude - Suchipakari Lodge (-1.0194136 S, -77.5978758 W, 390m) and Wildsumaco Biological

Station Sumaco, Napo (-.674638889 S, -77.60886111 W, 1500m) respectively. These sites were selected for their accessibility, and to capture a clear separation of altitude that is within (or just outside) the range of the two focal species (neither species is present at Wildsumaco, which is just above the altitudinal range of both species). Both sites were protected reserves with forest fragments embedded within a human-modified landscape containing undisturbed rainforest habitats with variable forest cover and temperatures.

Heliconius sara and *Heliconius erato* models were used in each experiment (Figure 1). Models were made using the wings of dead butterfly specimens (retrieved from insectary-reared stocks) attached with PVC glue to a 3cm x 1cm cardboard "body". Models were pinned through the cardboard to 4 polystyrene bases using entomological pins. Each base contained two *H. sara* and two *H. erato* models, with a total of 16 models used Wings were randomly allocated to each treatment on each day and replacement models were substituted if wings became damaged. Bases were positioned on the ground, in 4 different locations at each site, two in open areas and two in areas under the forest canopy. Each location was within a 100m radius of each other and had notably different levels of shading. The "cover" variable refers to the open and canopy-covered areas, selected for their accessibility and their differing exposure to sun/shade. We selected open sites that had no surrounding canopy, and wings would be exposed to full sun throughout the day. The closed positions were selected by the level of canopy cover, choosing areas where there was canopy cover whilst dappled light and occasional sunflecks still remained. The temperature of 6 regions on the wing was measured with a thermocouple (TMS 305P Type K, TC Direct) for each butterfly model, by gently holding the probe to the wing surface at a 90-degree angle. For each measurement point, three replicates were recorded, and averaged, to reduce measurement error. These measurements were taken throughout the day at one-hour intervals where possible, rotating between the four different locations and alternating between closed and open. Time of measurement and weather condition (sunny, cloudy or shaded - due to movement of the sun) was recorded. Recording was stopped during any periods of rain, and models had to be moved to avoid damage.

The air temperature before and after each set of measurements was taken by holding the thermocouple at waist height out of direct sun. The average between these two air temperatures was used to compare to the wing-surface temperatures for the analysis. The temperature of the wing surface was measured at the same position on the wing every time.

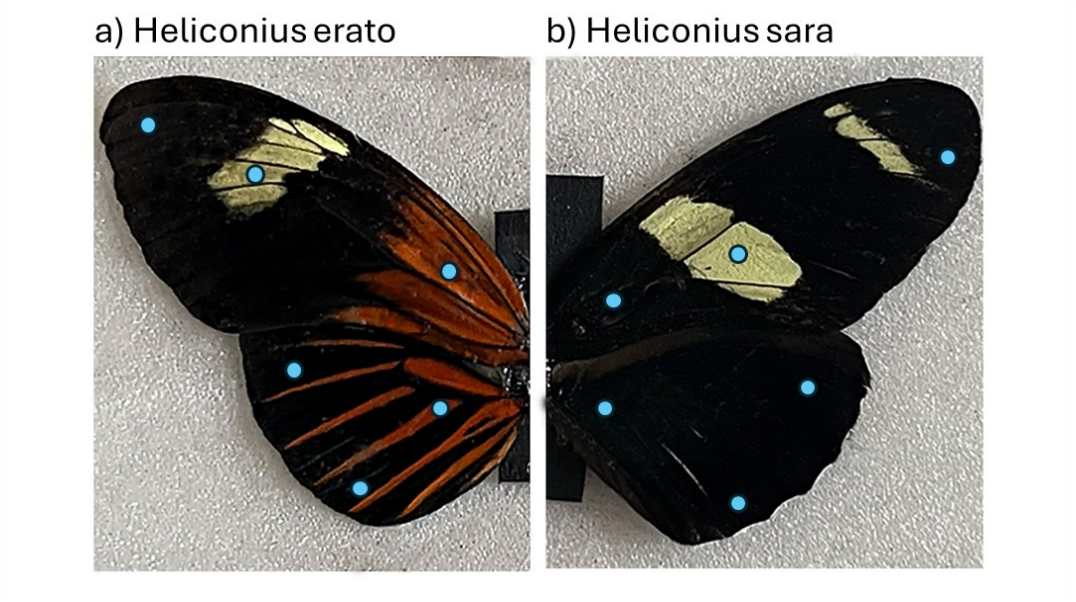


Figure 1: An example of butterfly "models", the images show just the left side of the model for a) *H. erato* and the right side of the b) *H. sara* model. The blue spots represent the area of the wing that were measured with the thermocouple.

Live Body Temperature: Experimental Design and Data Collection

Live butterflies were sampled using a net from a range of sites across multiple elevations in Eastern Ecuador between March to October 2021. Sampling sites were: San Pedro de Arajuno (-1.09759 S, -77.58389 W; altitude = 400m), Suchipakari Lodge (-1.025694444 S, -77.60475 W; altitude = 400m), Y de Misahualli (NA S, NA W; altitude = 400m), Venecia (-1.059833333 S, -77.70338889 W; altitude = 400m), Campococha (-1.092111111 S, -77.54627778 W; altitude = 400m), Capirona (-1.08207 S, -77.70982 W altitude = 400m), Universidad Regional Amazónica Ikiam (-0.948557 S, -77.86605 W; altitude = 600m), La Mina Negra (-0.720027778 S, -77.7470833 W; altitude =1300m), Finca Narupa bridge (-0.72516 S, -77.76736 W; altitude =1150m) (Supplementary Table 1). These

sites allowed the collection of samples across low- and mid-altitudes. The low altitude sites were grouped between 350- 600m and the mid altitude sites had an altitudinal range of 1100-1300m. Species were caught using a hand net and the temperature of the thorax was immediately (within 30 seconds) measured with a thermocouple by gently pressing the thermal probe to the thorax through the net, taking care not to touch any part of the butterfly. Air temperature was measured at the location of capture following the thorax temperature measurements, by holding the thermocouple at waist height out of direct sunlight. We also recorded whether the butterfly was flying in the open or under forest cover when it was first observed. Butterflies were then placed in glassine envelopes and transported back to the lab for detailed identification and weighing. All butterflies were weighed alive using a digital balance at the end of the same day on which they were caught. Some individuals were retained for other studies while some were marked (with Sharpie pen on the wings), to avoid recapture of the same individuals, before being released back at their capture site (the next day). Sampled individuals were photographed and identified to species level, verified by *Heliconius* experts, who know how to distinguish these species from their co-mimics. For *H. erato* this is primarily the red hind-wing "ray" pattern, which is distinct from the most common co-mimic, *Heliconius melpomene,* with body colour or other wing pattern features distinguishing it from other co-mimics. For *H. sara* the pattern of small red spots on the base of the ventral hind-wings can be used to distinguish it from *Heliconius congener*, which is occasionally also found in this area.

Statistical Modeling and Model Selection

Model selection was conducted using mixed effects models with the lmerTest package (Kuznetsova et al., 2017) for the wing temperature, and linear models (R version 4.5.2; R Core Team, 2025) for the live body temperature. AICc was applied to the list of models to test for the most parsimonious and best fitted model using the AICmovdag package (Mazerolle, 2023). This approach was used to balance model fit with model complexity, and to avoid overfitting through inclusion of additional interactions and variables that did not improve the model performance. As several predictors were biologically plausible, we considered multiple models. This approach allowed us to assess alternative

combinations of predictors and interactions to be compared based on their relative support from the data (Supplementary Table 2-4). As wings were randomly rotated between sites throughout the experiment, the wing ID and weather condition was included as a random effect in all statistical analysis performed on wing temperatures.

Analysis of the temperature difference between colours on the dead wing was conducted by fitting a series of models where colours were modelled against black wing regions as the reference. Model selection on the dead wing temperature included variables of surface colour, altitude, cover and air temperature (Supplementary Table 2). To focus on the environmental effects only, we separately modelled the dead wing temperature of the black regions of the wings against altitude, cover and air temperature. Wing temperature used in this analysis was averaged across each black region of each wing model, resulting in two black measurements per given time point. AIC model selection was conducted on models with dead wing temperature as the response variable, average air temperature, species, altitude (as a categorical variable) and cover (as a categorical variable) as predictor variables and with wingID and weather condition as random effects to control for wing replication and weather (Supplementary Table 3). Similarly, a series of linear models with the live body temperature as the response variable were fitted for an AIC model selection to select the best fitting model. Linear models on live body temperature included species, air temperature, altitude (as a categorical variable) and cover (whether the butterfly was flying in the open or under forest cover) and body weight (Supplementary Table 4). We used ANOVA comparisons to compare the top 4 models and determine the best model. As sample size for *H. sara* was low, we analysed and conducted an AIC selection on *H. erato* data only, to determine whether removal of *H. sara* would significantly affect the model.

The model selection for both dead wing and live body temperature (Supplementary Tables 3 and 4) included models where air temperature was set to a slope of 1. This allowed us to test whether there were significant deviations from thermo-conformity (as specified in Angilletta., 2009), as a thermal conformer would be expected to show a slope of 1 when modelling live or dead temperature against

ambient temperature. For models with ΔAIC < 2, further testing using nested ANOVA comparisons were performed to determine the best model for interpretation.

The emtrends function (Lenth & Piaskowski, 2025) was used to calculate an estimated marginal trend to test whether the slopes of the top-ranking AIC model significantly differ from 1. The function used (emtrends) takes the intercept of the variables from the model into account when calculating the slopes. This allowed us to determine whether the slopes of wings or body were significantly different from a 1:1 trend with air temperature.

4.4 Results

For wing temperatures, we had a total of 896 measurements (n= 448 *H. erato*, n= 448 *H. sara*), and a total of 183 (n= 159 *H. erato*, n= 24 *H. sara*) for live body temperatures (Supplementary Table 1). Across our dead wing temperature experiments, we captured air temperatures of 16.75°C - 30.20°C for the low altitude site, and 15.80°C - 24.20°C for the mid altitude site. Across our live body temperature measurements, air temperature at low altitude ranged from 20.9°C - 31.5°C and 18.7°C - 31.8°C at mid altitude. The measured live temperatures ranged from 21.5 °C - 37.3°C (*H. erato*) and 21.5 °C - 32.3°C (*H. sara*). Dead wing temperatures ranged from 15.6°C - 37.9 °C (*H. erato*) and 14.3°C - 35.5 °C (*H. sara*).

The effect of colour on passive wing temperature:

Using dead mounted wings, we compared the temperature across different wing surface colours, in relation to the ambient air temperature. Here, the top-ranking model included species, altitude, air temperature, cover and surface colour and a significant three-way interaction between altitude, cover and air temperature on the dead wing temperature (Table 1). The second ranking model including the interaction between surface colour and air temperature was marginally less well supported (ΔAIC = 4.87, Supplementary Table 2), suggesting a possible small effect of colour on the relationship between wing and air temperature. The surface wing temperature in blue and red regions was not significantly different from that of black surfaces (Table 1). The yellow wing regions, however, were very slightly,

but significantly cooler than black, red and blue regions of the wing (Figure 2A). Whilst this was significant in the model, the difference in temperature was very small (average yellow= 22.39°C, average black = 22.57°C) across all sites and locations (Figure 2 A). Additionally, there was a significant effect of species seen in the model, however these differences were also minimal (Figure 2 A).

Across all the different wing colour patches we observed very similar relationships between wing and air temperature. Higher air temperatures were associated with higher wing temperatures (Figure 2 B). At low altitude the air reached higher maxima than at mid altitude, and subsequently, there was a wider range of dead wing temperatures in both closed and open conditions at low altitudes (Figure 2 B). The relationship between wing and air temperature had less variance in closed conditions compared to open conditions in both mid and low altitude. These results illustrate the interaction between solar exposure (cover) with altitude and air temperature, which has a significant effect on wing temperature.

Table 1: The top AIC ranked mixed effects model explaining the effect of wing temperature across red, blue and yellow patches compared to black as the reference surface colour with wing identity and weather condition as a random effect. The altitude is a factor with two levels of mid (1500m) and low (390m). Cover refers to the open cover compared to closed cover as a reference value. Air temperature is the average of the temperature measured before and after each data measurement. The significant model variables are in bold.

*wingT ~ species + altitude * airT * cover + surface_colour*

	Estimate	**Std.error**	**Df**	**t-value**	**P-value**
(Intercept)	3.130	0.397	3542	7.89	<0.001
Species	**-0.181**	**0.071**	**156**	**-2.53**	**0.012**
altitude	**-3.819**	**0.838**	**3549**	**-4.56**	**<0.001**
airT	**0.868**	**0.017**	**3551**	**52.50**	**<0.001**
coveropen	**-5.923**	**0.518**	**3521**	**-11.43**	**<0.001**
surface_blue	0.078	0.074	1828	1.06	0.289
surface_red	0.035	0.079	2811	0.45	0.654
surface_yellow	**-0.135**	**0.061**	**3168**	**-2.23**	**0.026**
altitude:airT	**0.197**	**0.045**	**3545**	**4.39**	**<0.001**
altitude:coveropen	**5.669**	**1.022**	**3551**	**5.55**	**<0.001**

airT:coveropen	**0.319**	**0.0212**	**3499**	**15.05**	**<0.001**
altitude:airT:coveropen	**-0.220**	**0.053**	**3549**	**-4.15**	**<0.001**

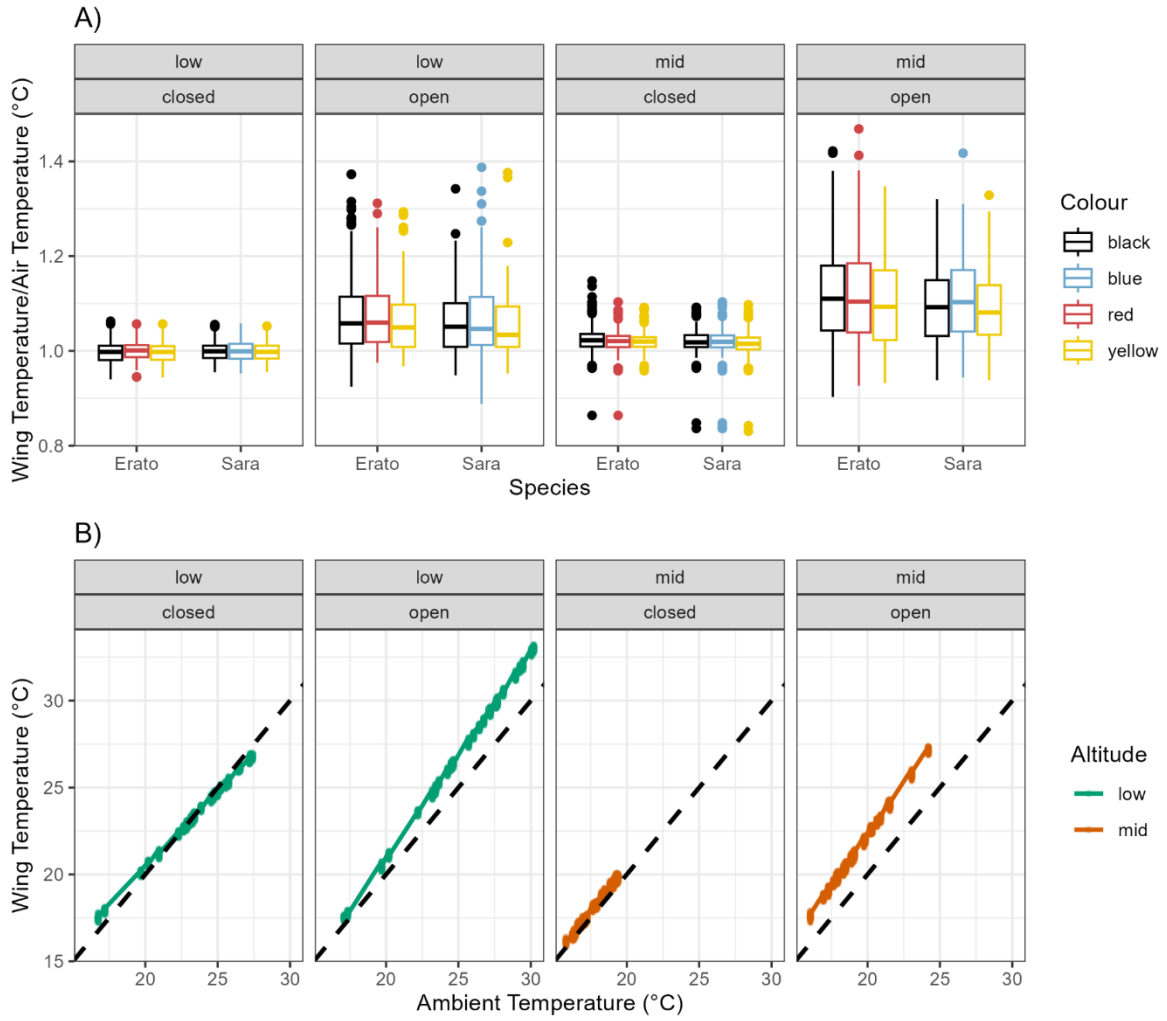


Figure 2 A: The air temperature against wing temperature relative to air temperature for each wing colour area in *H.erato* and *H.sara* at mid and low altitudes with open and closed cover. B: the relationship between wing and air temperature for all patches across mid and low altitudes with open and closed cover. The observed data points are plotted with lines predicted from the linear model in Table 1. The dashed line represents the expectation under thermoconformity: slope = 1.

The effect of altitude and cover on dead wing temperature:

To further investigate the interactive effects of air temperature, altitude and cover, irrespective of colour, we focussed on the black regions of the wings only. All models subsequent to this top-ranking model had a ΔAIC > 40 suggesting no or little statistical support (Supplementary Table 3). Wing temperature of the black wing regions was significantly influenced by species, air temperature, altitude and cover, as well as the interaction between three variables (air temperature, altitude and cover) (Table 2). Generally, higher air temperature was associated with higher wing temperatures (Figure 3). Importantly, wing temperature under canopy cover was lower (reduced solar exposure) compared to in the open (higher solar exposure) (Figure 3). The relationship between black-region wing temperatures and air temperature was significantly different between cover conditions, in interaction with altitude. In the open, wings at mid altitude (1500 masl) were on average warmer than wings at the same ambient air temperature at lower altitude (390 masl). In contrast, under canopy cover wing temperature increased with air temperature similarly in mid and low altitudes, suggesting the altitudinal effects were contingent on solar exposure (Figure 3).

The slopes of the relationships between wing and air temperature illustrate these patterns. If the wings only gained and lost heat through exchange with the surrounding air, we may expect a slope equal to one, which would indicate the air and wing temperature are increasing at the same rate. We ran a test of estimated marginal trends to determine whether the model slopes significantly differed from 1. This averages the slopes over other variables in the model such as species and tests a slope of the change in wing temperature (response) per unit of air temperature for groups of altitude and cover (alt~cover). Overall, in both mid and low altitude, when in open conditions the slope was significantly greater than one (low altitude: slope: 1.186, std.error: 0.0224, df: 1329, t.ratio: 8.319, p-value: <0.0001, mid altitude: slope: 1.156, std.error: 0.0423, df: 1288, t.ratio: 3.696, p-value: 0.0002), indicating that the wings warmed faster than the air. In closed conditions, however, this was different. At low altitude the slope was significantly less than one (slope: 0.868, std.error: 0.0282, df: 1335 t.ratio: -4.682, p-value: <0.0001), suggesting that wings warmed slower than the air temperature, and in mid altitude,

closed conditions, the slope was not significantly different from 1, indicating that the wings warmed at the same rate as the air (slope: 1.070, std.error: 0.0714, df: 1321, t.ratio: 0.982, p-value: 0.3261). This shows that in exposed solar conditions (i.e., the open cover) wings are susceptible to rapid heating, and where solar exposure is lower (i.e., under closed canopy cover), the wing temperature conforms more closely to the air temperature.

There were significant differences between *H. erato* and *H. sara* wings, although the same general trends were seen across altitude, cover and air temperature in both species (Table 2, Figure 3). Although significant, this effect of species was small in magnitude.

Table 2: The top-ranking AIC mixed effects model explaining wing temperature (black regions) changes with air temperature, altitude and cover types with wing identity and weather condition as a random effect. The altitude is a factor with two levels of low (390m) and mid (1500m) altitudes. Cover refers to the open cover compared to closed cover as a reference value. Air temperature is the average of the temperature measured before and after each data measurement. The significant model variables are in bold.

*wingT~ airT *alt_cat * cover + Species*

	Estimate	**Std. Error**	**df**	**t value**	**Pr(>|t|)**
(Intercept)	3.030	0.7934	888	3.82	<0.001
airT	**0.870**	**0.033**	**888**	**26.24**	**<0.001**
altitude	**-3.648**	**1.683**	**888**	**-2.17**	**0.031**
coveropen	**-5.219**	**1.0361**	**888**	**-5.04**	**<0.001**
Species	**-0.153**	**0.092**	**888**	**-1.67**	**0.095**
airT:altitude	**0.190**	**0.090**	**888**	**2.10**	**0.036**
airT:coveropen	**0.291**	**0.042**	**888**	**6.86**	**<0.001**
altitude:coveropen	**4.989**	**2.050**	**888**	**2.43**	**0.015**
airT:altitude:coveropen	**-0.196**	**0.107**	**888**	**-1.84**	**0.066**

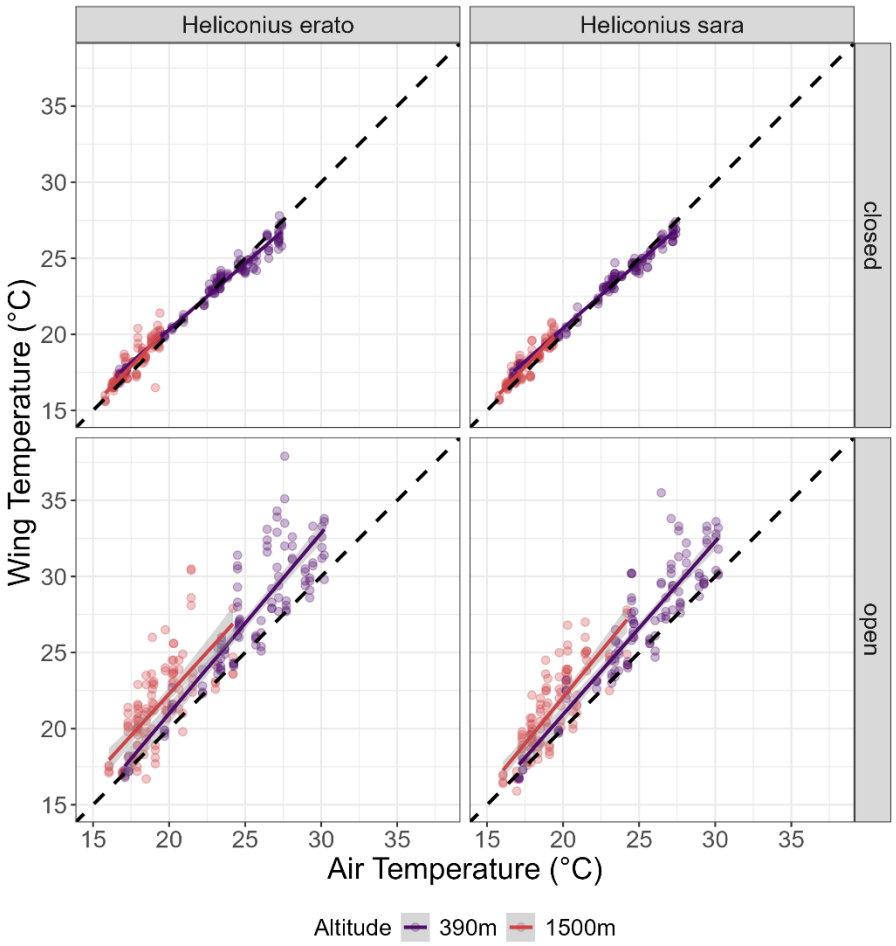


Figure 3: The wing temperature against ambient air temperature at open and closed cover. Raw data points are coloured by altitude, where purple is low (390m) and red is mid altitude (1500m). Closed refers to the wings under canopy cover (low solar exposure) and open refers to wings out of canopy tree cover (under direct solar exposure). The wing temperature is for the black region of the wings only and the ambient temperature is the average of the air temperature measured before and after each data measurement. Lines are predicted from the mixed effects model in Table 2 and observed data points are plotted below. The dashed line represents the expectation under thermoconformity: slope = 1.

Body Temperature in live butterflies:

In a second experiment we measured the thoracic temperature of live *H. erato* and *H. sara* butterflies caught under similar conditions to those in which we measured the dead wing temperatures. We

observed that the thoracic temperature of live butterflies was significantly affected by the interaction between air temperature and altitude (Table 3). Here, the top-ranking model included Air Temperature, Altitude and the interaction between Air Temperature and Altitude. There were 3 additional models with a ΔAIC < 2, showing similar support (Supplementary Table 4). Adding species or body weight as a model term or including a three-way interaction between species, air temperature, and altitude, did not significantly improve model fit, as tested with ANOVA comparisons (Supplementary Table 7). Additionally, the cover was not retained in the top model (Delta AICc = 3.976). Therefore, thorax temperature was best explained by the interactive effects of ambient air temperature and altitude.

As expected, thorax temperature increased with air temperature, but the strength of this relationship varied with altitude (Figure 4). Live body temperatures were generally higher than ambient air and, relative to air temperature, the body temperature was generally higher at low altitude compared to mid altitude. This contrasts with the wing results, where (in open conditions) wings were warmer at mid than low altitude, relative to air temperature. As the sample size for *H. sara* was low, particularly at mid altitude, we repeated the analysis with *H. sara* removed. This produced very similar results (Supplementary Table 5, 6), and the top AIC model remained the same as the analysis with *H. sara* included. As with the wing temperature, we tested whether the estimated slopes at mid and low altitude significantly differed from 1. Importantly, the body temperature was consistently higher than the air temperature, however the slope of the relationship varied with altitude. At mid altitude, thorax temperatures increased more slowly with air temperature, meaning thorax temperature rose by around 0.5 °C for each 1 °C increase in ambient temperature. This was significantly different from a slope of 1 where air and live body temperature would increase at the same rate (slope: 0.497, std. Error: 0.0913, df: 157, t.ratio: -5.505, p-value: <0.0001). At low altitude, thorax temperature more closely tracks air temperature, but was still significantly different from a slope of 1 (slope: 0.793, std.error: 0.0668, df: 157, t.ratio: -3.099, p-value: 0.0023).

These results are different from those found for the dead wings, which were warmer at mid altitudes than at low altitudes. At both low and mid altitudes, wings warmed faster than the air and the key difference in wing temperature was seen between open and closed conditions. Butterfly bodies however, warmed slower than the air temperature and were warmer at low altitudes than at mid altitude particularly at higher air temperatures. Here, altitude was again a key variable, where at low altitude, increases in body temperature tracked air temperature, and at mid altitude, bodies warmed significantly slower than the air.

Table 3: The top AIC ranked linear model explaining changes in body temperature with altitude and air temperature. The altitude is two categories that span ranges of low (350-600m) and mid (1100 – 1300m) altitudes. Significant model terms are in bold. The dashed line represents the expectation under thermoconformity: slope = 1.

*thoraxT ~ ambientT * altitude*

	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	8.699	1.712	5.08	<0.001
airT	**0.792**	**0.066**	**11.95**	**<0.001**
altitude	**6.137**	**2.727**	**2.25**	**0.026**
airT:altitude	**-0.295**	**0.113**	**-2.62**	**0.010**

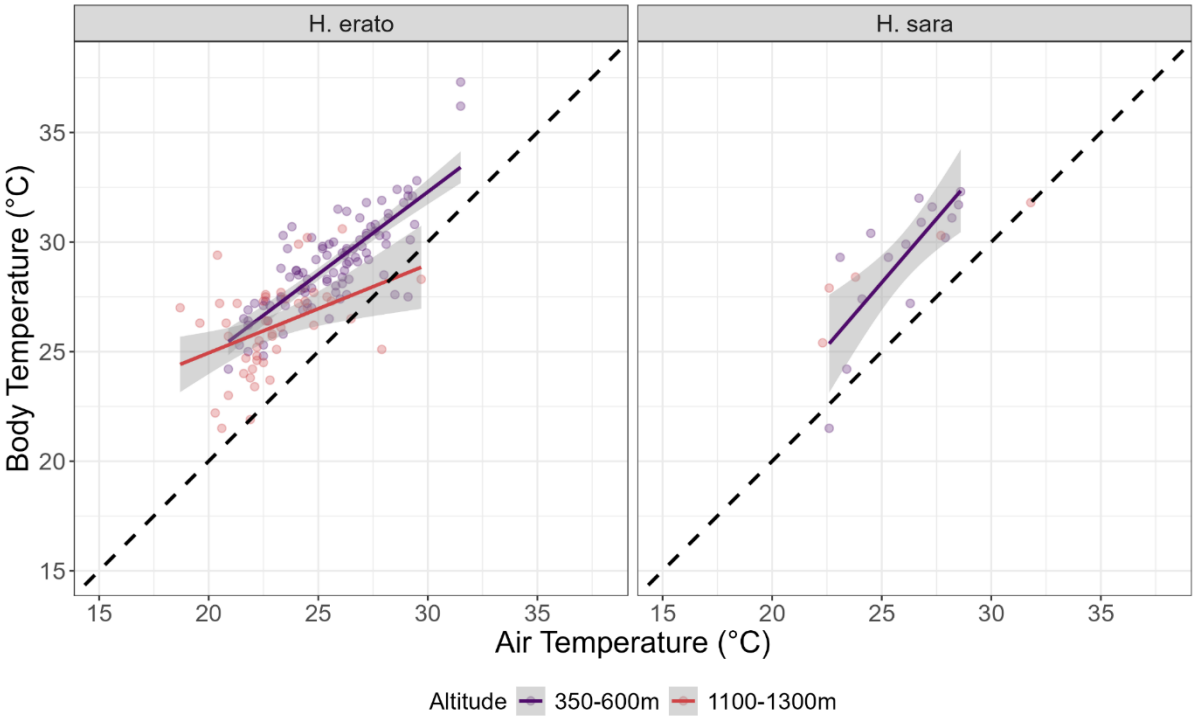


Figure 4: The body temperature against ambient air temperature. Points are coloured by altitude, where purple is low (390-600m) and red is mid altitude (1100-1300m). Lines are predicted from the linear model in Table 3 and observed data points are plotted. A line for mid altitude in *H. sara* is not plotted due to a lack of data.

4.5 Discussion

In this study, we found that dead wing surface temperature did not differ substantially among black, red, and blue regions, although yellow regions were slightly cooler. In contrast, environmental conditions had a strong influence on wing temperature. The relationship between wing and air temperature differed with forest cover, with wings heating above air temperature in open conditions but more closely tracking air temperature under canopy cover. Dead wing temperature was also strongly affected by altitude, with wings in open conditions at mid altitude becoming warmer than

those similarly exposed at low altitude, despite lower ambient temperatures. Live body temperatures showed a different pattern, remaining relatively stable across air temperatures and generally above ambient temperature. This effect was strongest at mid altitude, where thoracic temperature increased more slowly than air temperature. These results indicate that the live butterflies are able to maintain more stable body temperatures, and thoracic warming is less tightly coupled to air temperature at mid altitudes compared to low altitudes. Across all results there was little difference between *H. erato* and *H. sara*. Together, these results show that altitude has an important effect on both live butterfly bodies and dead wings, in differing ways. The passive heating of wings, outside of behavioural thermoregulation, is strongly influenced by altitude and solar exposure, likely due to differences in solar radiation. Live butterflies actively regulate thoracic temperature across different altitudes, and potentially exploit the increased solar radiation at higher altitudes to maintain body temperatures where ambient temperatures are cooler. Contrary to expectations, we found deadwing surface temperature did not differ significantly between black, red, or blue regions. Only yellow regions were slightly cooler than the other colours, and the magnitude of this effect was small. Previous studies have found that structural and pigment-based features of butterfly wings mediate passive heat gain and loss through convective and reflective properties (Kettlewell, 1973; Tsai et al., 2020; Krishna et al., 2020). For example, both phaeomelanin and eumelanin absorb solar radiation and convert it into heat (Riley, 1997; Meredith and Sarna, 2006). The thermal properties of ommochrome pigments responsible for red colouration are less well understood. However, the lack of differentiation between melanin-based colours and red suggests that red ommochrome pigments may absorb infrared wavelengths in a similar way to melanin (Nijhout, 1997; Kayser, 2013; Stavenga et al., 2014). In contrast, the 3OHK pigment responsible for yellow colouration does not absorb infrared wavelengths (Tokuyama et al., 1967; Reed et al., 2007; Finkbeiner et al., 2017; Stavenga et al., 2014). This likely explains why yellow regions were cooler, as the 3OHK pigment (responsible for yellow) likely reflects or transmits infrared radiation. Although wing nanostructures can reduce heat gain through surface reflection, we found no significant difference in temperature between black and iridescent blue patches (Tsai et al., 2020; Krishna et al., 2020). Together, these results suggest that within these

species, structural and pigment-based differences in visible wing colour do not strongly influence surface heating.

Environmental factors played a much stronger role in determining dead wing temperatures than live body temperatures. We found the relationship between wing temperatures and ambient air temperature was contingent on both altitude and forest cover. Under forest cover, wing temperature closely tracked air temperature, whereas in the open, wing temperature deviated from air temperature at both altitudes. In open conditions, wings were significantly warmer at mid altitude than at low altitude, at a given air temperature. In open habitats, wing temperature increased faster than air temperature, with slopes greater than one. Under closed cover, wing temperature increased more slowly, with slopes closer to or below one. High altitude environments typically experience lower air temperatures, but higher solar radiation compared to low altitudes (Vignola et al., 2019; Montejo Kovacevich et al., 2020; Blumthaler et al., 1997; Liou, 2002; Iqbal, 2012). Forest cover further modifies solar exposure, as open sites receive more direct radiation while closed forests are more shaded (Anderson, 1970; Lee, 1987; Royer et al., 2011). Although closed forests remain heterogeneous due to sunflecks, they experience lower overall radiation than open forests (Becker et al., 1989; Pearcy et al., 1990; Breshears et al., 1997). We found that the effect of forest cover was strongest at mid altitude, where open conditions amplified solar heating more than at low altitude. At low altitude, slopes were more similar between cover types, indicating a reduced influence of forest cover.

These results highlight solar exposure as a key driver of passive wing heating. Despite lower air temperatures at higher altitudes (Average low altitude = 24.4°C , Average mid altitude = 18.4°C), wings under open conditions reached higher temperatures than at lower altitudes, which we believe is most likely due to higher solar radiation. Differences in cloudiness and humidity with altitude could also potentially influence the amount of radiation reaching the earth's surface through scattering and reflecting solar radiation (Schneider, S. H. 1972; Matuszko, D. 2012; Becker et al., 1989). Humidity could also have affected dead wing temperatures through effects on heat exchange mediated by

evaporative and convective processes (Thornton and Running, 1999; Swartman & Ogunlade, 1967; Rathod et al., 2016). Whilst cloudiness was controlled for in our models, we cannot completely rule out its possible influence. We therefore cannot rule out an additional effect of cloud cover and humidity across different altitudes also impacting the heating of dead wing surfaces.

However, our results clearly highlight that solar exposure is an important consideration when interpreting the thermal responses of butterflies. We found that exposure to open versus closed conditions had a strong effect on passive wing temperature, with wings in open conditions heating above air temperature, whereas under canopy wing temperature more closely tracked air temperature. This effect was particularly pronounced at mid altitude, where wings exposed to open conditions were warmer than those at low altitude at equivalent air temperatures. Thus, our results suggest that variation in solar exposure can alter the relationship between ambient air temperature and passive wing heating, and that air temperature alone may not adequately describe the thermal environment experienced by butterfly wings. Solar exposure should therefore be considered in future studies of butterfly thermal responses, particularly in tropical systems. It is important to note, the *Heliconius* species we studied are generally darker in colour than many butterfly species, and so they could be absorbing more solar radiation than would be seen in other species. Our results provide support for the importance of considering solar exposure when interpreting thermal patterns across altitude. Our findings suggest that variation in solar exposure can alter the relationship between altitude, ambient temperature and wing temperature, and may help explain why traits that reduce solar absorption, such as wing transparency (Ossola et al. 2024), could become more prevalent at higher elevations despite lower ambient temperatures. However, direct measurements of solar radiation across altitudes would be needed to test this hypothesis.

Live body temperatures showed very different patterns from those seen in dead wings. Thoracic temperatures did not show thermal conformity (Angilletta, 2009) and did not mirror trends observed in dead wings. If butterfly temperature was solely determined by the ambient temperature, we would expect the wing and body temperature to be lower at mid altitudes. However, at lower air

temperatures the dead wings were 1°C - 4°C higher than those at lower altitudes. In contrast, live body temperatures of the butterflies at mid altitude were 1°C - 5°C lower than those from low altitudes, as expected with lower air temperature in mid altitudes. This suggests that at low air temperatures, the mid altitude butterflies could use the increased solar radiation to passively increase body temperature, but we find limited evidence for this since live butterfly bodies showed reduced body temperatures at mid altitudes. However, at mid altitude, thorax temperature increased more slowly than air temperature, rising by roughly 0.5 degrees for each 1 degree increase in ambient temperature. At low altitude, thorax temperature had a slope closer to one, and more closely tracked the increases in air temperature, but remained consistently higher than ambient conditions. Although morphological differences are known to mediate such thermal buffering (Defour et al. 2018, Bladon et al., 2020; Laird Hopkins et al., 2023), the large difference we observe between the passively heating dead wing and the live bodies strongly indicates active thermoregulation in the live butterflies.

Active thermoregulation in butterflies includes basking, shade seeking, evaporative cooling through respiration, and increased haemolymph circulation (Clench, 1966; Kingsolver and Moffat, 1982; Kingsolver and Koehl, 1985; Heinrich, 1993; Kemp and Krockenberger, 2004; Ashe Jepson et al., 2023; Lahondere, 2023; Bladon et al., 2020; Laird Hopkins et al., 2023). We found that live body temperatures were consistently above ambient temperatures to an extent not seen in the passively heated dead wings, most likely suggesting that there is endothermic heat produced by the flight muscles (Kammer 1970; Heinrich, 1993; Clench, 1966; Kingsolver, 1988). The thorax does have a smaller surface area to volume ratio so will lose heat less rapidly than the wing, but we think this is unlikely to be sufficient to explain the higher temperature in live thoraxes compared to dead wings relative to air temperature, particularly given that the wings were placed in direct sunlight.

These findings may also have implications for how butterflies respond to a warmer future climate. As ambient temperatures increase, the capacity for behavioural and physiological thermoregulation may become increasingly important, particularly in open habitats where solar exposure can substantially increase wing temperatures above ambient conditions. However, passive thermal properties of the

wings may also provide an important mechanism for buffering environmental change. Wing colour, pigmentation, structural features and surface properties can influence the amount of solar radiation absorbed, reflected or transmitted by the wing, and therefore the amount of heat gained without requiring active energy expenditure. These properties could potentially provide a relatively consistent thermal buffer across changing environmental conditions, reducing reliance on behavioural thermoregulation when temperatures become stressful. Conversely, traits that increase solar absorption may be beneficial in cooler environments by facilitating rapid heat gain but could become disadvantageous as temperatures increase. This suggests that selection on wing optical and structural properties could potentially shift under climate warming, favouring traits that reduce heat gain or increase heat dissipation in populations increasingly exposed to high temperatures. In particular, species or populations that are able to alter the balance between passive heat gain and loss through wing properties may have greater capacity to cope with changing thermal environments. These effects are likely to interact with behavioural thermoregulation and habitat structure, as the availability of shaded microhabitats may determine whether butterflies can compensate for increased passive heat gain.

Alternatively, our results that solar radiation can produce considerable additional heat gain even when air temperatures are relatively low, meaning that increases in both air temperature and solar exposure could also increase the risk of overheating. Butterflies may partly buffer these effects through behaviours such as shade seeking and movement between exposed and sheltered microhabitats, as well as through physiological mechanisms that increase heat loss. However, if environmental temperatures increasingly approach or exceed the upper thermal limits of these species, opportunities to behaviourally avoid heat may become more important and potentially more constrained. This could be particularly relevant in tropical forests where changes in canopy structure and increased exposure following forest disturbance could alter the balance between solar heat gain and shade availability. Conversely, at higher elevations, where butterflies currently experience cooler air temperatures but potentially high solar exposure, warming may reduce the thermal benefit of basking while increasing the risk of overheating during periods of direct exposure. Although our study cannot predict how

these responses will change under future climate conditions, our findings highlight that assessments of butterfly vulnerability to warming should consider solar exposure and fine-scale thermal environments alongside air temperature alone.

Endothermic heat production depends on metabolic activity and may be constrained in cooler environments (Kingsolver and Koehl, 1985), as low ambient temperatures can suppress metabolic rates, limiting heat generation even under high solar radiation (Mellanby, 1997; Kearney et al., 2009; Carter et al., 2025; Lahondere, 2023). Therefore, endothermic heat generation alone is unlikely to explain the strong departure from thermal conformity that we observed. At both altitudes the butterflies are likely using solar radiation to increase their body temperature at lower air temperatures through basking, contributing to the relatively warmer body temperatures at the lowest air temperatures (Barton et al., 2014; Kemp and Krockenberger, 2004). At high air temperatures the risk of overheating may be mitigated through behavioural and physiological control, including shade seeking and increased convective heat loss via enhanced haemolymph flow (Clench, 1966; Heinrich, 1993; Kemp and Krockenberger, 2004; Kingsolver and Moffat, 1982; Kingsolver and Koehl, 1985). This would explain why the slopes of the relationships between live body and air temperature were less than one (thermal conformity) and flatter than those seen for the passively heating dead wings. Warmer conditions may also promote behavioural thermoregulation such as movement between shaded and open habitats. The shallower slope at the generally cooler, mid altitude could suggest that under these conditions, the live butterflies can more effectively lose heat as the air temperature warms, or gain heat less at these higher temperatures. In addition, at cooler, mid altitudes, the butterflies likely have a greater need to gain heat at the cooler air temperatures and may do this through increased active behavioural thermoregulation. This may include utilising increased solar radiation at higher altitudes to warm more at the lowest air temperatures. Whilst we cannot distinguish between these processes with our results, we show that live butterfly thermoregulation differs significantly from passive thermoregulation experienced in the dead wings.

Interestingly, we did not find an effect of body weight on live thorax temperature despite having a range of 0.15g - 1.03g. There is an expectation that lower surface to volume ratio in larger butterflies would result in slower warming and cooling rates compared to smaller individuals (Heinrich, 1993) and several previous studies have reported body size as an important predictor of thermal buffering in butterflies (Ashe Jepson et al., 2023; Bladon et al., 2020). However, those studies focussed on between-species variation with larger ranges in body size, whilst our study was within-species with a relatively low variance of body size (variance = 0.11g). It is also likely that the substantial environmental variation in our study would have swamped any effect of small differences due to body size, which may require controlled laboratory conditions to detect.

Our results demonstrate that solar exposure associated with canopy cover can substantially modify dead wing temperature, however there was an absence of an effect of canopy cover in live butterflies. This result is somewhat unexpected for heliothermic butterflies, for which increased exposure to direct sunlight might be expected to increase solar heat gain (Ohsaki, 1986; Kemp and Krockenberger, 2004; Heinrich, 1993). One possible explanation is that butterflies recorded under canopy may have previously moved between shaded and exposed locations. We were only able to record where they were first observed, and this may be a poor predictor of how much time they spent in the sun in the relevant time-window before capture. Butterflies are known to behaviourally thermoregulate and move between sun and shade to maintain a constant body temperature (Barton et al., 2014; Clench, 1966; Kingsolver, J. G., 1985), so a butterfly caught in the shade may have recently been basking in the sun. In addition, our study may also have had limited power to detect differences between butterflies caught in the forest versus open conditions as we had uneven sampling with the majority of butterflies caught in the open (Open = 124, Closed = 38).

The altitudinal pattern observed in our dead wings may have broader implications for understanding thermal adaptation. High-elevation environments are often characterised by lower ambient temperatures but can experience greater solar irradiance, meaning that solar exposure could potentially offset some of the thermal effects of cooler air temperatures. In our study, however, solar

irradiance was not directly measured, and therefore we cannot determine whether differences in irradiance caused the higher wing temperatures observed at mid altitude. Other environmental variables that vary with elevation, including cloud cover, humidity and wind speed, may also contribute to differences in heat exchange. Additionally, this study had a relatively short sampling period, with measurements collected over nine days. This restricts the range of environmental conditions represented. The relationships observed here may not capture the full extent of seasonal variation in thermal environments, as variation in solar irradiance, cloud cover, humidity and wind speed across seasons could alter the relative importance of passive heating and behavioural thermoregulation. For example, periods of greater solar exposure may amplify differences in wing temperature between open and closed conditions, whereas lower solar exposure could reduce these differences. Similarly, seasonal changes in environmental conditions may alter the extent to which butterflies rely on behavioural thermoregulation to maintain thoracic temperature. Longer-term sampling across different seasons and weather conditions would therefore be valuable for determining whether the patterns observed here are consistent across a broader range of environmental conditions.

Overall, live and dead butterflies were neither strictly passive thermal conformers nor fully active thermoregulators. Under thermal conformity the assumption is that dead wing and live body temperatures should increase in direct proportion to air temperature, with slopes of one (Angilletta, 2009). Following our experiments with dead wings, the null expectation would be that in open conditions body temperatures should increase faster than air temperature, due to absorption of solar radiation, and that this effect is greater at higher elevation. Instead, thorax slopes were consistently below one, i.e. more stable, particularly at higher altitude, providing strong evidence for thermoregulation. A key distinction between dead wings and live bodies was the relative influence of air temperature and solar radiation. Dead wing temperature was strongly affected by solar exposure, leading to relatively higher wing temperatures at cooler mid altitudes than at warmer low altitudes, something that when alive butterflies are likely to exploit through basking. This may also be a way live butterflies are able to elevate their body temperatures at cooler air temperatures. Live body temperature was highest in the warmer low altitude environments, suggesting that the increased solar

radiation at higher altitudes had minimal effect on the body temperature of the live butterflies, particularly at warmer air temperatures. These patterns support the interpretation that butterflies rely on both passive and active mechanisms to regulate dead wing and live body temperatures differently, with solar radiation likely playing an important role in passive heating.

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Data Availability: The data is available from https://figshare.com/s/ad52e8bfdbccf00425b6

Diversity and Inclusion Statement: Our study is the result of an equitable international collaboration involving researchers based in both the UK and Ecuador, representing a range of career stages from research assistants to principal investigators. Authors collaboratively contributed diverse perspectives which were considered throughout the entire project. Where relevant, literature published in both

English and Spanish was revised and cited. We are committed to open and accessible science, and all data, code, and supplementary materials associated with this study will be made openly available.

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4.7 Supplementary Figures:

Supplementary Table 1: A table of the collection sites for body temperature measurement, and the sample sizes for each species.

Site Name	Coordinates	Altitude (m)	H. erato sample n=159	H. sara sample n=24
San Pedro de Arajuno	-1.09759 S, -77.58389 W	400	7	0

Suchipakari Lodge	-1.025694444 S, -77.60475 W	400	17	4
Y de Misahualli	NA S, NA W	400	16	0
Venecia	-1.059833333 S, -77.70338889 W	400	27	2
Campococha	-1.092111111 S, -77.54627778 W	400	2	0
Capirona	-1.08207 S, -77.70982 W	400	15	4
Universidad Regional Amazónica Ikiam	-0.948557 S, -77.86605 W	600	28	9
La Mina Negra	-0.720027778 S, -77.7470833 W	1300	6	0
Finca Narupa bridge	-0.72516 S, -77.76736 W	1150	41	5

Supplementary Table 2: The AICc analysis for the best model fit for a series of 11 linear mixed effects models analyse the temperature of different wing colours, with wing temperature as the response variable and the wing identity and weather condition as a random effect. Models were built using lmerTest R package (ref) and AICc analysis performed in the AICmovdag package (ref). The top model is in bold. Abbreviations for modelled variables are as followed: WT= wing temperature, AT= air temperature, ALT= category of altitude, COV= open or forest cover, SP= species, SURF= surface colour.

Models	K	AICc	Delta_AICc	ModelLik	AICcWt	LL	Cum.Wt
WT~SP+ALT*AT*COV+SURF	**14**	**12298.12**	**0**	**1.00E+00**	**9.19E-01**	**-6134.998**	**0.9192914**
WT~SP+ALT*AT*COV+SURF*AT	17	12302.98	4.865517	8.78E-02	8.07E-02	-6134.404	1
WT~SP+ALT*AT+COV+SURF	11	12519.85	221.734647	7.09E-49	6.52E-49	-6248.888	1
WT~SURF+ALT+AT+COV+SP	10	12522.92	224.800395	1.53E-49	1.41E-49	-6251.427	1
WT~SP+ALT*AT+COV	8	12524.52	226.408292	6.86E-50	6.30E-50	-6254.242	1
WT~SURF+ALT+AT+COV	9	12527.35	229.23312	1.67E-50	1.54E-50	-6254.649	1
WT~SP*AT*ALT	10	13450.28	1152.165306	6.46E-251	5.94E-251	-6715.109	1
WT~SURF+ALT+AT	8	13512.94	1214.827414	1.60E-264	1.47E-264	-6748.451	1
WT~SP*AT	6	13718.62	1420.502872	0.00E+00	0.00E+00	-6853.297	1
WT~SP*ALT	6	19003.11	6704.996211	0.00E+00	0.00E+00	-9495.544	1
WT~SURF	6	20395.81	8097.689852	0.00E+00	0.00E+00	-10191.891	1

Supplementary Table 3: The AICc analysis for the best model fit for a series of 19 linear mixed effects models with wing temperature on the black region of the wings, as the response variable and the wing identity and weather condition as a random effect. Models were built using lmerTest R package (ref) and AICc analysis performed in the AICmovdag package (ref). The top model is in bold. Abbreviations for modelled variables are as followed: WT= wing temperature, AT= air temperature, ALT= category of altitude, COV= open or closed forest cover, SP= species.

Models	K	AICc	Delta_AICc	ModelLik	AICcWt	LL	Cum.Wt

WT~AT*ALT*COV+SP	**11**	**3098.495**	**0**	**1.00E+00**	**1.00E+00**	**-1538.097**	**1**
WT~AT+ALT*COV+SP	7	3141.401	42.90586	4.82E-10	4.82E-10	-1563.637	1
WT~AT+ALT+COV+SP	8	3141.942	43.4469	3.68E-10	3.68E-10	-1562.889	1
WT~AT*ALT+COV+SP	8	3142.255	43.75979	3.15E-10	3.15E-10	-1563.046	1
WT~offset(AT)+ALT+COV+SP	6	3150.379	51.88355	5.42E-12	5.42E-12	-1569.142	1
WT~ offset(AT)+COV+SP	5	3163.776	65.281	6.67E-15	6.67E-15	-1576.854	1
WT~AT+COV+SP	6	3164.943	66.44754	3.72E-15	3.72E-15	-1576.424	1
WT~AT+COV	5	3165.469	66.97368	2.86E-15	2.86E-15	-1577.701	1
WT~AT*ALT+SP	6	3377.507	279.01136	2.59E-61	2.59E-61	-1682.706	1
WT~AT*ALT	7	3377.909	279.41385	2.12E-61	2.12E-61	-1681.891	1
WT~SP*AT*ALT	10	3382.821	284.32559	1.82E-62	1.82E-62	-1681.285	1
WT~AT+ALT+SP	5	3393.714	295.21822	7.84E-65	7.84E-65	-1691.823	1
WT~AT+ALT	6	3394.168	295.67253	6.24E-65	6.24E-65	-1691.036	1
WT~offset(AT)+ALT+SP	4	3446.485	347.98941	2.72E-76	2.72E-76	-1719.22	1
WT~AT+SP	5	3447.106	348.61014	2.00E-76	2.00E-76	-1718.519	1
WT~AT	4	3449.876	351.38087	5.00E-77	5.00E-77	-1720.916	1
WT~offset(AT)+ALT	5	3451.024	352.52885	2.81E-77	2.81E-77	-1720.478	1
WT~SP*AT	6	3453.013	354.51708	1.04E-77	1.04E-77	-1720.459	1
WT~SP*ALT	6	4743.523	1645.02762	0.00E+00	0.00E+00	-2365.714	1

Supplementary Table 4: The AICc analysis for the best model fit for a series of 17 linear models with thoracic (body) temperature as the response variable. Models were built using the lm function in R and AICc analysis performed in the AICmovdag package (ref). The top model is in bold. Abbreviations for modelled variables are as followed: BT= body temperature, AT= air temperature, ALT= category of altitude, SP= species, W= weight, COV= whether the individual was caught in the open or under cover.

Models	**K**	**AICc**	**Delta_AICc**	**ModelLik**	**AICcWt**	**LL**	**Cum.Wt**
BT~AT*ALT	**5**	**615.4515**	**0**	**1.00E+00**	**3.55E-01**	**-302.5334**	**0.3553746**
BT~SP*AT*ALT	9	616.595	1.143485	5.65E-01	2.01E-01	-298.7054	0.5559982
BT~AT*ALT+SP	6	616.7785	1.326977	5.15E-01	1.83E-01	-302.1183	0.7390344
BT~AT*ALT+weight	6	617.5991	2.147554	3.42E-01	1.21E-01	-302.5286	0.8604714
BT~AT*ALT+COV	7	619.4274	3.97585	1.37E-01	4.87E-02	-302.35	0.9091504
BT~AT+ALT	4	620.1972	4.745719	9.32E-02	3.31E-02	-305.9712	0.9422762
BT~AT*ALT+COV+SP	8	620.7417	5.290198	7.10E-02	2.52E-02	-301.9003	0.9675072
BT~AT+ALT+SP	5	621.9809	6.529397	3.82E-02	1.36E-02	-305.7981	0.9810856
BT~AT+ALT+weight	5	622.3173	6.865768	3.23E-02	1.15E-02	-305.9663	0.9925619
BT~AT*ALT*COV	10	624.3221	8.870591	1.19E-02	4.21E-03	-301.4326	0.9967737
BT~AT*ALT*COV+SP	11	625.7138	10.262289	5.91E-03	2.10E-03	-300.9769	0.9988738
BT~AT	3	627.8601	12.408632	2.02E-03	7.18E-04	-310.8541	0.9995919

BT~AT+SP	4	629.6616	14.210104	8.21E-04	2.92E-04	-310.7034	0.9998837
BT~SP*AT	5	631.5015	16.049965	3.27E-04	1.16E-04	-310.5584	1
BT~offset(AT)+ALT	3	648.0283	32.576746	8.43E-08	3.00E-08	-320.9382	1
BT~offset(AT)+ALT+SP	4	650.111	34.659527	2.98E-08	1.06E-08	-320.9281	1
BT~SP*ALT	5	728.6813	113.229809	2.58E-25	9.19E-26	-359.1484	1

Supplementary Table 5: The AICc analysis on *H. erato* only, for the best model fit for a series of linear models with thoracic (body) temperature as the response variable. Models were built using the lm function in R and AICc analysis performed in the AICmovdag package (Mazerolle, 2023). The top model is in bold. Abbreviations for modelled variables are as followed: BT= body temperature, AT= air temperature, ALT= category of altitude, SP= species, W= weight, COV= whether the individual was caught in the open or under cover.

Models	**K**	**AICc**	**Delta_AICc**	**ModelLik**	**AICcWt**	**LL**	**Cum.Wt**
BT~AT*ALT	**5**	**529.9182**	**0**	**1.00E+00**	**6.37E-01**	**-259.739**	**0.636909**
BT~AT*ALT+weight	6	532.0721	2.153908	3.41E-01	2.17E-01	-259.725	0.85386
BT~AT*ALT+COV+weight	7	534.091	4.172745	1.24E-01	7.91E-02	-259.628	0.932923
BT~AT+ALT	4	535.3561	5.437907	6.59E-02	4.20E-02	-263.532	0.974923
BT~AT+ALT+weight	5	537.3662	7.447927	2.41E-02	1.54E-02	-263.463	0.990297

BT~AT*ALT*COV+ weight	10	538.3455	8.427261	1.48E-02	9.42E-03	-258.333	0.999719
BT~AT	3	545.3675	15.44923	4.42E-04	2.81E-04	-269.597	1
BT~offset(AT)+ALT	3	565.6206	35.70236	1.77E-08	1.13E-08	-279.723	1

Supplementary Table 6: The top AIC ranked linear model for *H. erato* only, explaining changes in body temperature with altitude and air temperature . The altitude is two categories that span ranges of low (350-600m) and mid (1100 – 1300m) altitudes. Significant model terms are in bold. The dashed line represents the expectation under thermoconformity: slope = 1.

*thoraxT ~ ambientT * altitude*

	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	9.763	1.73944	5.613	<0.001
airT	**0.7508**	**0.06748**	**11.126**	**<0.001**
altitude	**7.11775**	**3.01§397**	**2.362**	**0.0196**
airT:altitude	**-0.34782**	**0.12638**	**-2.752**	**0.0067**

Supplementary Table 7: The ANOVA comparisons comparing models to the top AIC model (BT~AT*ALT) Abbreviations for modelled variables are as followed: BT= body temperature, AT= air temperature, ALT= category of altitude, SP= species, W= weight.

Model	F-value	P-value
BT~AT*ALT (Comparison model)		
BT~SP*AT*ALT	1.86	0.12
BT~AT*ALT+SP	0.81	0.37
BT~AT*ALT+weight	0.21	0.81

5.0 Iridescence reduces infrared emissivity in butterfly wing scales

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5.1 Abstract:

Butterfly wing optics are typically interpreted through visible reflectance and their infrared properties remain poorly understood. As butterfly wings play an important role in thermoregulation, understanding the infrared optical properties is an important factor in assimilating wing material thermal properties and the ecological consequences of this. We compared transmission, reflectance, and emissivity between melanic, and iridescent scales, compared to the bare wing membrane, to assess how pigmentation and nanostructure shape radiative behaviour. While reflectance remained consistently low across visible and infrared wavelengths, transmission varied substantially among scale types and emerged as the primary determinant of emissivity. Membranes exhibited high transmission and relatively uniform spectral behaviour, whereas melanic scales showed reduced transmission and elevated infrared emissivity consistent with strong broadband absorption by melanin. In contrast, iridescent scales displayed significantly lower infrared emissivity than melanic

scales, and in some cases lower than membranes, despite often containing embedded melanin. These differences between iridescent and melanic scales were strongly wavelength-dependent and associated with pronounced non-linear spectral structure, consistent with thin-film interference within multilayer nanostructures. Our results demonstrate that colour-producing structures can emit and absorb infrared wavelengths differently from pigmented colour patches, which is specific to visible and infrared wavelengths. This illustrates the importance of the scale structure in modulating radiative heat exchange in butterfly wings.

5.2 Introduction:

Butterfly colouration arises from both pigmentary and structural mechanisms (Duncan, 1940). Structural colour is often produced by multilayer chitin–air arrangements within scales, where thin-film interference occurs due to phase differences between light reflected from successive interfaces (Kinoshita et al., 2008; Ghiradella, 1991, 1984; Vukusic & Sambles, 2003). Constructive or destructive interference depends on optical path differences governed primarily by layer thickness and refractive index contrast, resulting in angle-dependent colouration. Even small changes in layer thickness can produce large shifts in reflectance, because they alter the optical path length through the film and therefore the wavelengths that undergo constructive and destructive interference. As a result, thinner films generally reflect less incident light than thicker films (Miaoulis & Heilman, 1998; Edalatpour & Francoeur, 2013; Tchenka et al., 2025). However, most work on these multilayer systems focuses on visible wavelengths, with comparatively limited consideration of infrared interactions, despite their potential importance for thermal regulation (Teolis et al., 2007; Stuart-Fox et al., 2017).

Radiative heat exchange in biological materials is governed by the interaction between reflection, transmission, absorption, and emission of electromagnetic radiation (Fox, 1953). According to Kirchhoff's law of thermal radiation, for a material in thermodynamic equilibrium, emissivity equals absorptivity at a given wavelength, meaning that surfaces that efficiently absorb radiation also efficiently emit it (Robitaille, 2009; Quintela et al., 2009). In turn, emitted radiative energy increases

with temperature according to the Stefan–Boltzmann law, where radiative power is proportional to temperature raised to the fourth power (Giddings, 2016; Montambaux, 2018). Accordingly, a material's optical behaviour is determined by how incident radiation is partitioned into reflected, transmitted, and absorbed components, which together sum to one (Robitaille, 2009; Pigeat et al., 1998).

In biological materials, transmission refers to the propagation of light through a medium, reflectance to the proportion of incident radiation reflected at a surface, and scattering to deviations in light trajectory caused by structural heterogeneity. Together, these processes determine how light interacts with complex nanostructures such as butterfly wing scales, influencing both visible colouration and infrared thermal behaviour (Pigeat et al., 1998; Guenther, 2018; Zhang, 2009). In Lepidoptera, the wing is covered by scales composed primarily of chitin (refractive index ~1.5–1.62) with many air-filled compartments (refractive index ~ 1.0), and this refractive index contrast is fundamental to optical effects such as thin-film interference.

In butterfly wings, the multilayer structures are formed by alternating chitin and air layers organised into ridges, cross-ribs, and laminae. Such nanostructures produce strong wavelength- and angle-dependent reflectance (Huxley, 1975; Ghiradella, 1991; Vukusic et al., 1999; Tada et al., 1999). Melanin is often embedded within these scale architectures, particularly in iridescent species such as *Morpho* spp., where it can enhance colour saturation by absorbing non-structurally reflected light (Ghiradella, 1984, 1991; Kinoshita et al., 2002; Giraldo & Stavenga, 2016). However, melanin is also a strong broadband absorber and may therefore influence thermal energy balance, particularly under solar irradiation (True, 2003; Trullas et al., 2007; Riesz & Jean, 2007; Biermann et al., 2002). Given its known thermoregulatory roles in other taxa, its function within structurally coloured wings may extend beyond optical enhancement alone.

Despite this, the infrared optical properties of butterfly wing nanostructures remain poorly understood. Butterflies rely on radiative heating and cooling for thermoregulation (Tsai et al., 2020), yet most studies of scale optics focus on visible wavelengths, with limited coverage of infrared ranges

(Krishna et al., 2020). This has contributed to unresolved and sometimes conflicting interpretations of whether photonic structures act primarily to enhance absorption or reflectance of solar energy. Some studies suggest multilayer structures increase absorption and may function as “solar collectors” (Biermann et al., 2002; Bosi et al., 2008), with examples including *Papilio palinurus* and *Archaeoprepona meander* (Heilman & Miaoulis, 1994; Berthier, 2005). In contrast, other work reports reduced absorption and increased reflectance associated with photonic crystal structures, particularly at shorter wavelengths (Koon & Crawford, 2000; Biró et al., 2003). Comparative analyses in butterflies, further suggest that while iridescent species may exhibit higher overall solar absorption than non-iridescent species, near-infrared reflectance can remain broadly similar across groups (Bosi et al., 2008), highlighting the complexity of these interactions.

In addition, the interaction between pigmentary and structural components remains poorly resolved. Melanin embedded within chitin-based photonic architectures may significantly modify broadband absorption, particularly in the infrared, yet its combined optical and thermal effects remain insufficiently quantified. This leaves a key gap in understanding how structural colour and pigmentation jointly influence energy balance in butterfly wings.

Here, we address the foundations of this gap by comparing the reflection, transmission, and emissivity of iridescent and black butterfly wing scales across visible and infrared wavelengths up to 2500 nm, in order to decouple the relative contributions of structural (iridescent) and pigmentary (brown) mechanisms to wing optical and thermal properties. Specifically, we aim to (1) quantify and directly compare the optical properties and emissivity of structural (iridescent) and pigmentary (brown) scales, (2) characterise the infrared emissivity spectrum of iridescent scales, and (3) determine whether emissivity varies with wavelength and whether this wavelength dependence differs between scale types.

As melanin is known for absorbing higher levels of infrared, it is expected that brown patches would have higher emission of infrared compared to the iridescent scales. As literature on the infrared optical properties of iridescent structures is contrasting, this study aims to characterise the infrared emission spectrum of iridescent scales. We also aim to make a direct comparison between structural

iridescent patches and brown pigmentary patches of the wing. We hypothesise that brown scales will have significantly different emissivity compared to iridescent scales, where brown scales would have higher infrared emissivity than iridescent scales. Additionally, to further understand the optical properties of brown and iridescent scales, we assess whether emissivity is wavelength dependent, or whether emissivity remains the same across all wavelengths. It is expected that there will be a level of wavelength dependence, as the optical properties of the wing are likely to have certain wavelength-specific characteristics, with the underlying scale structure and pigmentation influencing the interaction of different wavelengths with the wing surface. Together, these measurements allow us to assess whether structural colour patches have different emissivity from pigmentary colour patches and provide a basis for understanding how differences in wing material properties may contribute to thermoregulation.

5.3 Methods:

Species Selection

Nymphalid butterfly species that contained both iridescent and black patches on the wing were chosen: *Batesia hypochlora, Bia actorion, Eunica clytia, Eunica norica, Hamadryas arinome, Heliconius sara, Morpho helenor, Prepona pheridamas* (Figure 1). Selected species were spread across the Nymphalid phylogeny (with the exception of the two *Eunica* species) to enable the observation of general trends. Images of dry pinned museum specimens of male butterflies, from the scientific collections at the Natural History Museum London (NHMUK) were used in an initial analysis.

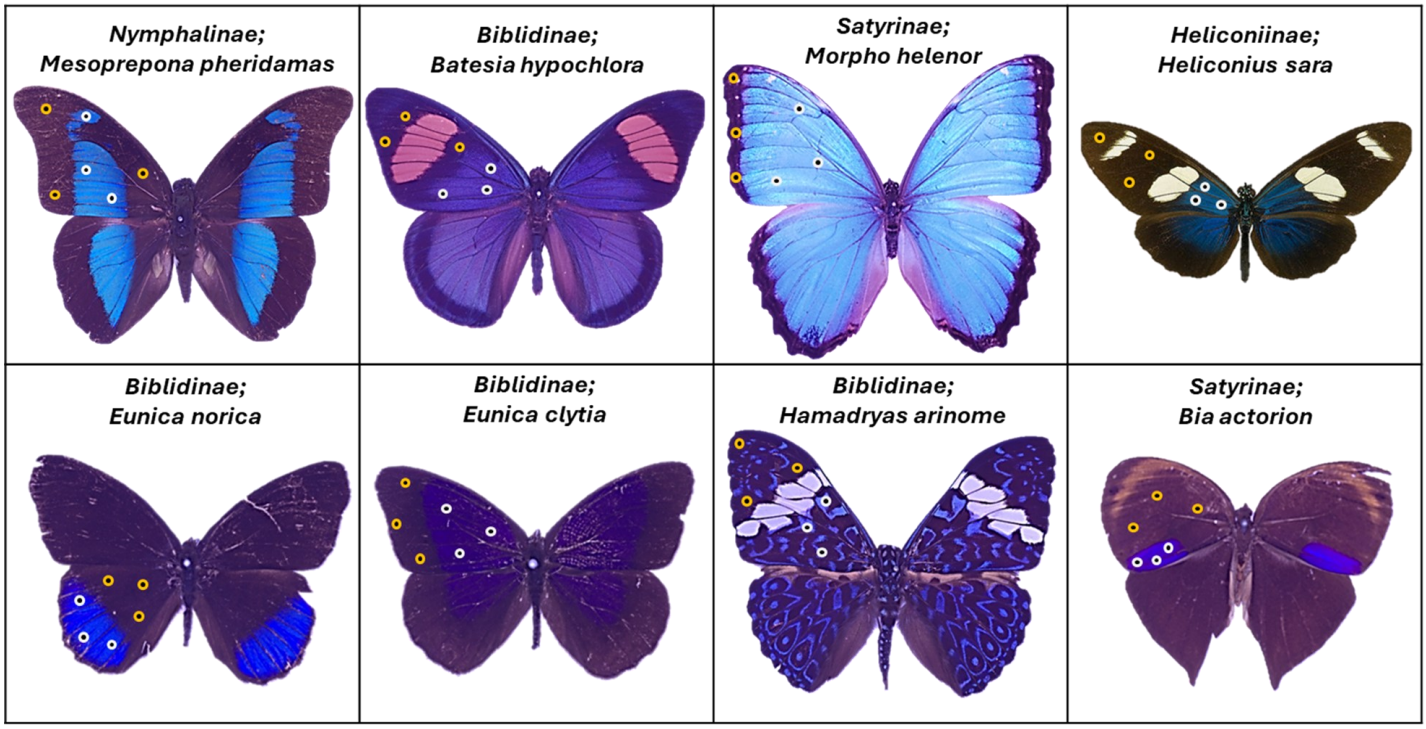


Figure 1: The images for each species used. The points indicate the measured brown (yellow points) and iridescent (white points) patches.

Dry pinned butterfly specimens were loaned from the Natural History Museum, London (except for *H. sara* and *M. helenor*, where samples were available from dead insectary-reared individuals), with permission to destructively sample. Specimens were prepared for spectroscopy by removing a single wing. To allow direct comparison of the optical properties of pigmentary and structural colouration, measurements were taken from brown scale patches and iridescent scale patches on the dorsal wing surface. To remove any optical effect of the ventral scales, these were removed from a small patch under the dorsal region of interest with a paintbrush. An adjacent scale-free patch was also prepared to quantify the contribution of the wing membrane independently of the overlying scales. To obtain measurements of the membrane only, scales were also removed with a paintbrush from both dorsal and ventral sides of an adjacent patch on the wing.

Reflectance, Transmission and Emissivity

To characterise the wavelength-dependent optical properties of pigmentary and structural colouration and calculate emissivity across the visible and infrared regions, the transmission and reflection of each wing specimen was measured using an Ocean Optics Spectrometer (IR 900-2500nm, and VIS 300-1000nm) with an Ocean Optics High-Power Tungsten-Halogen light source. For transmission the transmission standard was taken with nothing between the source and detector probes. For reflection the full reflectance standard used was aluminium (mirror-like reflector). For both visible and infrared, the transmission and reflection were measured at three points of the wing; three patches on the black regions and three patches on the iridescent regions (Figure 1) without the ventral scales. Measuring both regions across the same wavelength ranges allowed direct comparison of their optical properties and provided the reflectance and transmission measurements required to estimate emissivity. We also measured transmission and reflection for a single patch of the wing membrane on each wing with both dorsal and ventral scales removed, allowing the contribution of the membrane to be separated from that of the overlying scales. For each patch measurement, the spectrometer was set to take 3 measurements.

Spectral data was imported using the PAVO package in R (Maia et al., 2019) and spectral measurements for each patch and species were averaged, resulting in single transmission and reflectance measurement for each region type per species. Data was smoothed using the procspec function after these transformations.

The VIS and IR emissivity/absorptivity for each species was calculated using Kirchhoff's law of thermal radiation (Robitaille, 2009; Quintela et al., 2009), (equations 1-3). As our aim was to compare the optical and thermal properties of the scales themselves, rather than the combined scale–membrane surface, we corrected the composite emissivity measurements to account for the contribution of the wing membrane. We transformed the raw emissivity values (Supplementary Figure 1) using Equation 4. The emissivity/absorptivity of the dorsal brown and iridescent scales ($Emissivity_{scale}$) was calculated from measurements of the scales on the membrane ($emissivity_{composite}$) for the emissivity/absorptivity of the membrane ($emissivity_{membrane}$) using equation 4. This produced separate estimates of emissivity

for brown scales, iridescent scales and the membrane and enabled us to directly compare the emissivity of brown dorsal scales, iridescent dorsal scales and the membrane alone (Figure 4).

Statistical Modelling:

To test our hypotheses that (1) brown and iridescent scales differ in emissivity and (2) emissivity varies with wavelength, we fitted generalised additive models (GAMs) separately for the visible (Table 1) and infrared (Table 2) emissivity data. Brown patches were used as the reference level, allowing the emissivity of iridescent patches to be compared directly with that of brown patches. The models tested whether the overall emissivity of iridescent scales differed from brown scales and whether the relationship between emissivity and wavelength differed between scale types. Specifically, models included a global smooth, s(wl), tested for differences in the shape of the emissivity curves between brown and iridescent scales, and assessed whether the shape of the emissivity curves change non-linearly across wavelengths compared to the global (average) curve. Pairwise comparisons were then used to assess differences in emissivity between brown and iridescent scales across wavelengths.

$$Emissivity + transmission + reflection = 1 \quad (1)$$

$$Emissivity = absorption \quad (2)$$

$$Emissivity = 1 - (transmission + reflection) \quad (3)$$

$$Emissivity_{scale} = (emissivity_{composite} - emissivity_{membrane}) / (1 - emissivity_{membrane}) \quad (4)$$

5.4 Results:

Transmission and Reflection

The raw reflectance across the visible and the infrared spectra was minimal for all species (Figure 2). There was little difference between the reflectance of black and iridescent scales across both the visible and infrared wavelengths. The raw transmission was higher in the membrane (Figure 3), as expected as the membrane is a transparent material. In the visible spectra, all species membranes

exhibit a peak around 380nm, where transmission dips. For all but three species (*Batesia hyochlora, Bia actorion, Heliconius sara*), the visible transmission was higher in the iridescent scales compared to the brown melanin scales. Across all species and scale types, as wavelength increased there was a general increase in infrared transmission. For all species apart from *Bia actorion*, the infrared transmission was higher in the iridescent scales compared to the brown melanin scales. The key result here is that transmission has notable trends between scale surfaces whilst reflection has much less, suggesting emissivity/absorptivity is predominantly determined by light that is not transmitted through the wing, rather than by what is reflected.

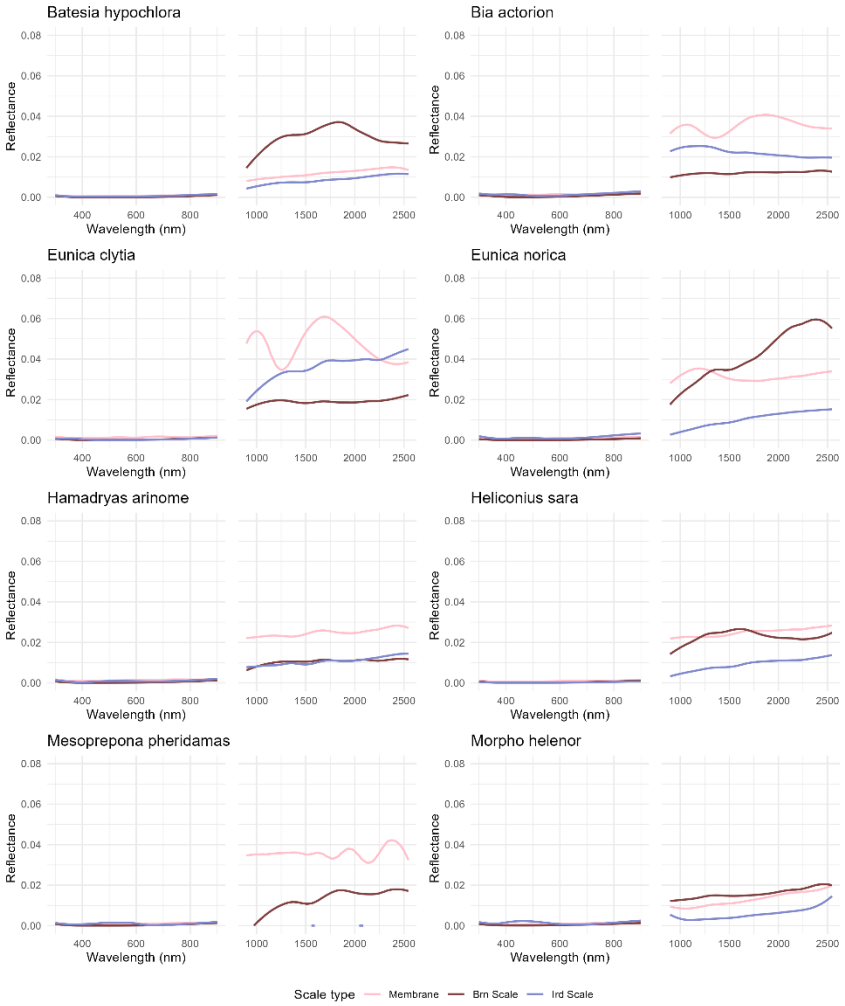


Figure 2: The visible (300-900nm) and infrared (900-2500nm) reflection across wavelengths for the wing membrane, and brown and iridescent scales (on the wing membrane), for each species.

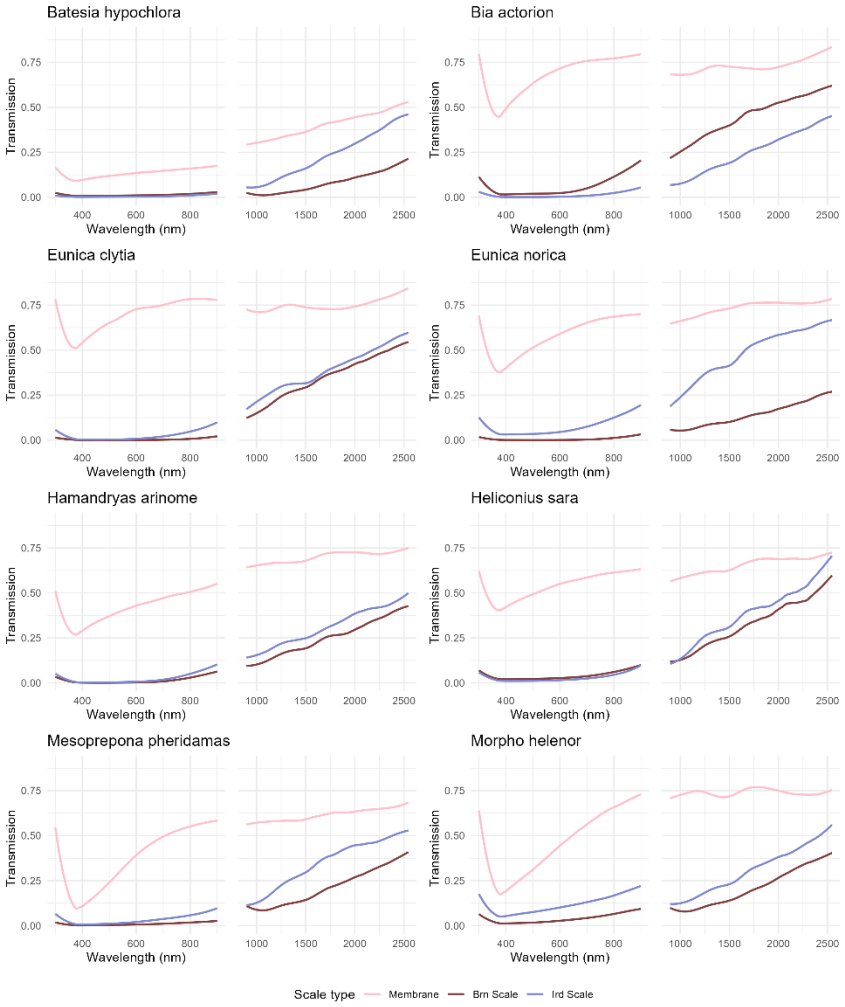


Figure 3: The visible (300-900nm) and infrared (900-2500nm) transmission across wavelengths for the wing membrane, and brown and iridescent scales (on the wing membrane), for each species.

Emissivity/absorptivity

The wing transmission affected the emissivity values more than the wing reflectance values. There was a difference in emissivity/absorptivity trends between surface types that shifted between the visible (300-900nm) and infrared wavelengths (900-2500nm). The visible emissivity/absorptivity of all species membranes exhibited a peak around 380nm, where the transmission curves dip (Figure 4; Figure 3). In the visible spectrum there was some separation between brown and iridescent emissivity/absorptivity however the differences were generally minimal (Figure 4). In the infrared spectrum the emissivity/absorptivity generally diverged more between brown and iridescent scales, where iridescent scale emissivity/absorptivity was generally lower in all species but *Bia actorion*. The

emissivity/absorptivity of infrared wavelengths of the iridescent scales in some instances, became lower than the emissivity/absorptivity of the membrane, suggesting that iridescent scales were less absorptive at particular infrared wavelengths, than the near transparent wing membrane. This shows there was a clear difference between the emissivities of brown and iridescent scales especially in the infrared wavelengths.

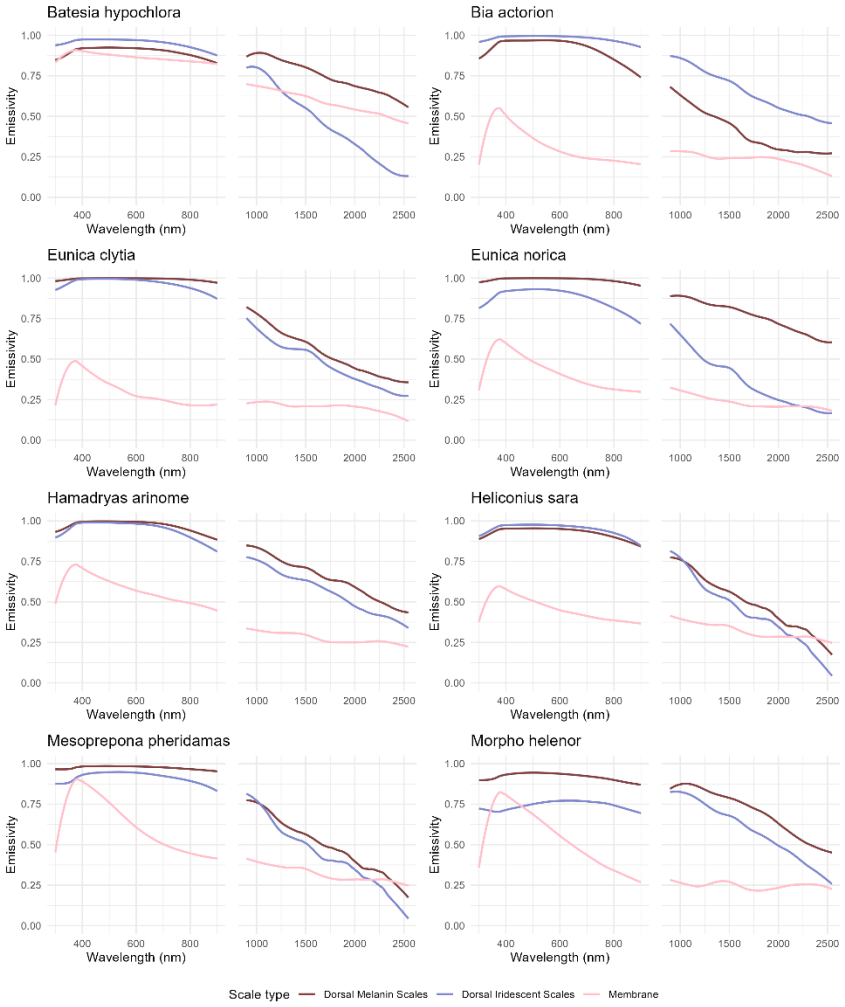


Figure 4: The calibrated visible (300-900nm) and infrared (900-2500nm) emissivity for the wing membrane, and brown and iridescent scales on the wing membrane, for each species.

Wavelength-Dependence of Emissivity

The GAM analyses showed that brown scales changed relatively smoothly across wavelengths, with little evidence of additional non-linear spectral structure in the infrared. In contrast, iridescent scales

displayed highly significant smooth terms and elevated effective degrees of freedom, indicating substantial wavelength-specific deviations from the global emissivity curve. Here, iridescent scales showed significantly lower emissivity across wavelengths. The additional smooth term for brown scales was not significant, suggesting that brown scales largely followed the shared global wavelength trend. In contrast, the smoothed terms for the iridescent scales showed significant curvature/non-linearity across wavelengths compared to brown scales (Table 1).

For emissivity in the infrared wavelengths (Table 2), the iridescent scales also had significantly lower emissivity than the brown scales. The smoothed term of the brown scales was again not significant, showing there was effectively no additional wavelength-dependent deviation for brown scales after accounting for the global curve. Iridescent scales had strongly significant smoothed terms, where iridescent scales had a strong non-linear wavelength dependence in the IR (Table 2; Supplementary Figure 3). This supported the trend seen in Figure 3, where brown decreased smoothly across wavelengths whilst the emissivity in iridescent scales was more wavelength dependent.

Pairwise comparisons revealed wavelength-dependent differences in both the visible and infrared ranges (Figure 5). In the visible spectrum, differences between scale types were generally small and varied non-linearly across wavelengths. The confidence intervals overlapped zero from below 400nm, and around 650nm and above, indicating limited and localized spectral divergence between brown and iridescent scales in the visible range (Figure 5a). In contrast, infrared emissivity showed a pronounced and consistent divergence between scale types across wavelengths (Figure 5b). Differences increased systematically across the infrared range, with iridescent scales exhibiting lower emissivity at shorter IR wavelengths and higher relative emissivity at longer wavelengths (Figure 5b). The confidence intervals indicated that these differences were more persistent and widespread than in the visible spectrum, demonstrating stronger spectral separation in the infrared region.

Table 1: The results of the GAM model on visible emissivity/absorptivity, showing the parametric (P Terms) and Smoothed (S Terms). Smoothed terms compare the curve to the global curve s(wl). The effective degrees of freedom (edf) show the degree of non-linearity of a curve.

Visible Parametric Terms

	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	0.949	0.001	1108.440	0.00E+00
Iridescent scale	-0.033	0.001	-27.163	1.32E-156

Visible Smooth Terms

	edf	**df**	**F**	**p-value**
s(wl)	6.988	7.968	138.052	0.00E+00
s(wl):surfaceEscale_brn	0.695	0.705	2.767	1.63E-01
s(wl):surfaceEscale_ird	2.471	3.146	14.529	0.00E+00
s(Species)	6.998	7	1314.5	0

Table 2: The results of the GAM model on infrared emissivity/absorptivity, showing the parametric (P Terms) and Smoothed (S Terms). Smoothed terms compare the curve to the global curve s(wl). The effective degrees of freedom (edf) show the degree of non-linearity of a curve.

Infrared Parametric Terms

	Estimate	**Std. Error**	**t value**	**Pr(>|t|)**
(Intercept)	0.620	0.001	650.110	<0.001
Iridescent scale	-0.121	0.001	-89.889	<0.001

Infrared Smooth Terms

	edf	**Ref.df**	**F**	**p-value**
s(wl)	8.423	8.625	83.201	<0.001
s(wl):surfaceEscale_brn	0.667	0.667	0.187	0.724
s(wl):surfaceEscale_ird	4.917	6.025	26.555	<0.001
s(Species)	6.998	7	1249.5	<0.001

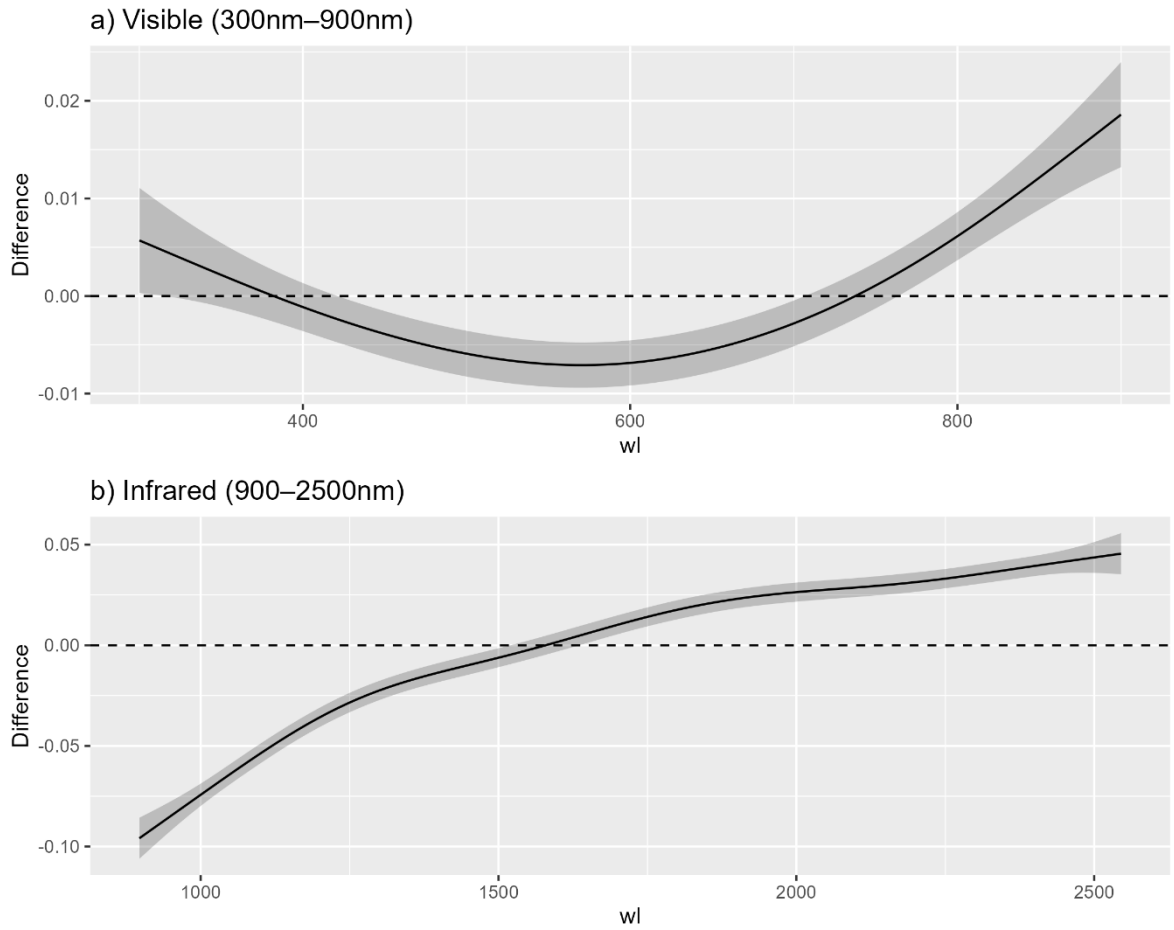


Figure 5: The estimated difference in infrared emissivity between iridescent and brown wing scales across a) visible wavelengths and b) infrared wavelengths. Differences were calculated as the predicted emissivity of iridescent scales minus that of brown scale, where negative values indicate emissivity of iridescent scales is lower than brown scales.

5.5 Discussion:

Butterfly wings perform multiple functions simultaneously, including visual signalling and the regulation of heat exchange with the environment. These functions are mediated by the optical properties of wing scales, yet the extent to which structures responsible for visible colour also influence infrared radiation remains poorly understood. Understanding this relationship is particularly important because butterflies rely heavily on radiative heat exchange for thermoregulation, meaning that wing structures involved in colour production may also influence thermal balance. We found that

between the scale types we measured there was more variation in transmission than reflection, suggesting that the thermal optical properties are governed more strongly by the passage of light through the wing than by surface reflection. Although we largely focussed on dark and melanic scales, there were noticeable differences in their visual appearance. This supports the idea that butterfly wing optics cannot be understood solely through visible colour appearance, but instead arise from complex interactions between pigmentation, multilayer nanostructure geometry, and wavelength-dependent interference processes (Ghiradella, 1984; Ghiradella, 1991; Vukusic & Sambles, 2003). The minimal differences in reflectance between brown and iridescent scales observed in our results indicate that visible appearance may conceal substantial differences in how energy is transmitted and absorbed within the wing.

We observed a strong role of transmission, a result that is consistent with thin-film interference theory. Butterfly scales are composed of alternating layers of chitin and air arranged into multilayered ridges and lamellae, where light is repeatedly reflected and refracted through nanoscale structures (Huxley, 1975; Ghiradella, 1991; Vukusic et al., 1999). As described by Miaoulis & Heilman (1998), even very small changes in film thickness can dramatically alter optical behaviour through constructive and destructive interference. Importantly, most previous studies focused primarily on reflected visible wavelengths, whereas the present results demonstrate that transmission through these thin films may have stronger implications for absorptivity and emissivity, particularly in the infrared spectrum. The observations here, that reflectance varied little whilst emissivity changed strongly with transmission, suggest that, for the wing surfaces examined here, differences in infrared energy exchange may be more strongly associated with wavelength transmission and absorption within the scale layers than with surface reflection.

The membrane consistently showed high transmission across wavelengths, as expected as the membrane is a relatively transparent chitinous substrate without any dense pigmentation or complex multilayer architecture. The visible transmission dipped around 380nm, which corresponded to the emissivity peak after calculation of emissivity, and reflects the intrinsic absorption properties of chitin and cuticular proteins as insect cuticles are highly absorptive around 290 to 330 nm (Ben-Yakir &

Fereres, 2016;) Because this feature occurred across nearly all species, it likely represents a conserved UV absorption property of butterfly wing membranes rather than species-specific structural adaptations.

The differences between brown and iridescent scales were much more pronounced in transmission than in reflectance. In most species, iridescent scales transmitted more visible and infrared radiation than brown melanic scales. This is consistent with the strong broadband absorptive properties of melanin (True, 2003; Trullas et al., 2007). Melanin-rich scales likely absorb a larger proportion of incident radiation within the scale itself, reducing transmission through the wing. This is supported by the elevated emissivity/absorptivity of brown scales seen here across infrared wavelengths. This finding therefore supports the hypothesis that melanin contributes directly to thermoregulatory heat absorption. This aligns with broader thermal melanism theory, where darker surfaces facilitate greater radiative heat gain.

The infrared properties of iridescent scales revealed a more complex interaction between pigmentation and nanostructure. Despite many iridescent scales containing embedded melanin within multilayer structures (Ghiradella, 1984; Ghiradella, 1991), their infrared emissivity was generally lower than that of brown scales and in some cases even lower than the membrane itself. In Morpho butterflies, melanin has previously been proposed to enhance visible iridescent saturation by absorbing scattered wavelengths outside the dominant reflected band (Huxley, 1975; Giraldo & Stavenga, 2016). Our results suggest that these same nanostructures may simultaneously influence infrared radiation in ways not previously considered. Rather than simply enhancing colour signals, multilayer photonic structures may also modulate the absorption and emission of heat.

We observed a strong wavelength dependence in the emissivity of iridescent scales, which further supports this interpretation. Brown scales showed relatively consistent emissivity across wavelengths, with little evidence of pronounced spectral variation in the infrared. In contrast, iridescent scales exhibited substantially greater wavelength-dependent variation, with distinct changes in emissivity across the spectrum, supporting a stronger influence of wavelength on the emissivity of iridescent

scales. Such complex spectral behaviour is consistent with the optical properties of interference-based structures, where interactions between reflected and transmitted wavelengths are sensitive to layer thickness, refractive index differences, and optical path distances (Miaoulis & Heilman, 1998; Vukusic & Sambles, 2003). Because thin-film interference is inherently wavelength dependent, the curvature observed in the infrared smooth terms likely reflects selective interactions between infrared radiation and the chitin-air multilayer nanostructures within iridescent scales.

These findings help address the limited understanding of how butterfly thin-film nanostructures interact with infrared wavelengths. Previous work demonstrated that thicker chitin-air layers can increase reflectivity in visible wavelengths (Miaoulis & Heilman, 1998; Lo & Lee, 2014), but infrared optical behaviour has remained largely unexplored. Here, the strong divergence in infrared emissivity between scale types suggests that multilayer nanostructures may substantially alter thermal radiation exchange. This suggests that butterfly iridescence may have an underappreciated influence on thermoregulation in addition to its established role in signalling and communication.

Butterflies rely heavily on radiative heat exchange for thermoregulation (Tsai et al., 2020), and small changes in infrared absorptivity could significantly alter wing heating dynamics. Lower infrared emissivity in iridescent scales may reduce radiative heat gain in exposed environments such as in forest canopies, potentially preventing overheating while still maintaining conspicuous structural colour signals. Conversely, brown melanic scales may favour greater heat absorption in cooler or more thermally variable habitats. The decoupling of visible reflectance from infrared emissivity therefore suggests that butterflies may evolve wing surfaces that independently optimise signalling and thermal performance across different regions of the electromagnetic spectrum.

The exceptional optical behaviour in species such as *Bia actorion*, which did not follow the general infrared trend, further highlights the diversity of structural solutions underlying iridescence. Iridescent colouration encompasses a wide variety of nanostructural geometries, and subtle differences in layer spacing or melanin incorporation may substantially alter infrared optical properties. Future work combining ultrastructural imaging, thermal measurements, and optical modelling would therefore help

determine which specific nanostructural arrangements are responsible for the reduced infrared emissivity observed here.

Overall, these findings demonstrate that butterfly wing infrared optical properties are shaped predominantly by wavelength-dependent transmission and absorption rather than surface reflection. More broadly, our results suggest that structural colouration should not be considered solely as a visual trait but also that the nanostructures that generate iridescence may also influence how butterflies exchange thermal radiation with their environment. This provides evidence that iridescent wing surfaces can function as multifunctional photonic structures, potentially linking visual signalling with thermoregulatory performance. The finding that iridescent and brown scales can have similar visible reflectance but substantially different infrared properties further suggests that the thermal consequences of wing colouration cannot necessarily be inferred from visible appearance alone. This has implications for understanding how butterflies may balance signalling and thermal requirements across different environments and highlights infrared optical properties as a potentially overlooked component of thermoregulation of butterfly wings.

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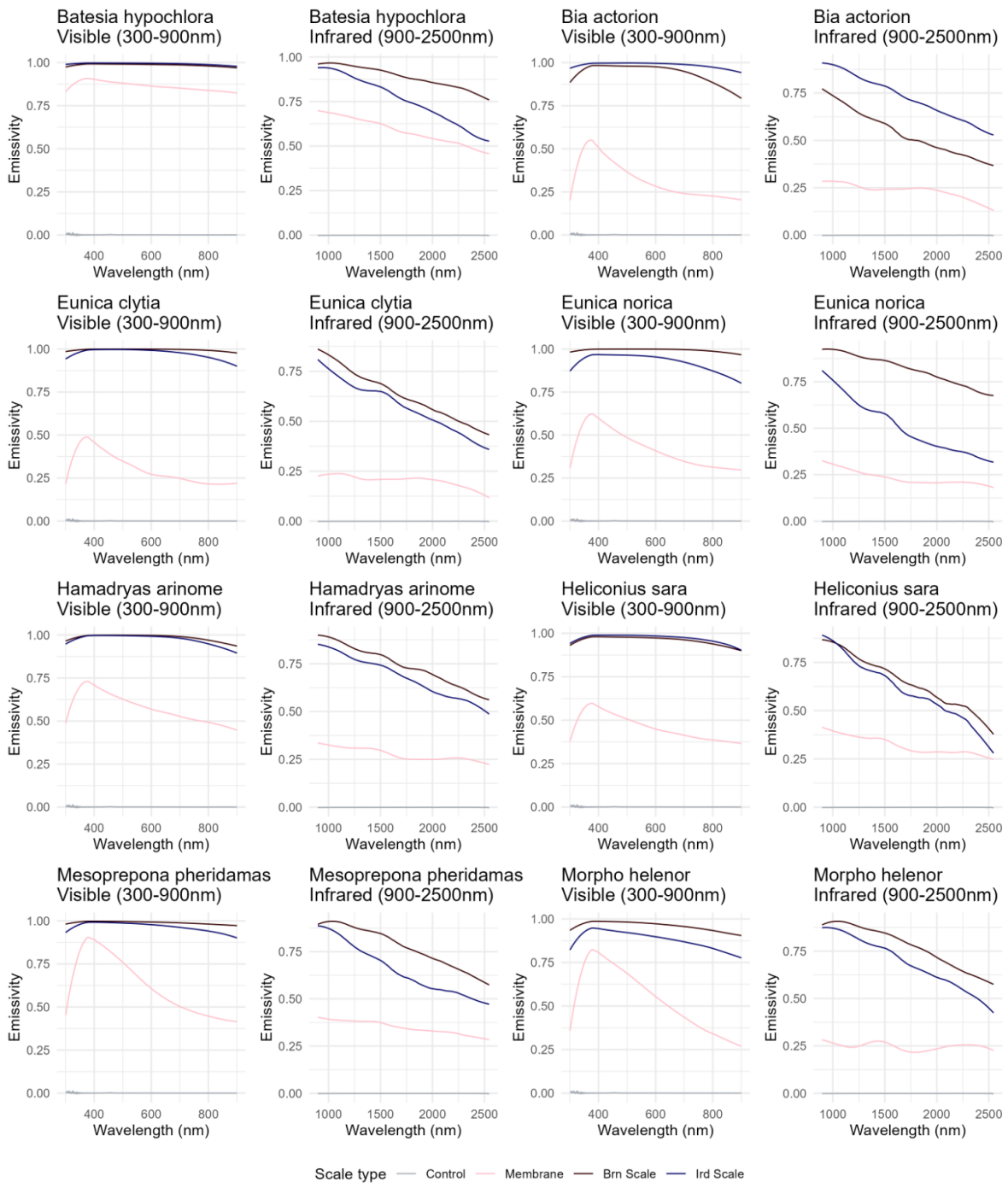
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5.7 Supplementary Figures:



Supplementary Figure 1: The non-transformed, visible and infrared emissivity/absorptivity curves for each species for the scales combined with the wing membrane.

6.0 Thesis Discussion

This thesis investigated how environmental variation across tropical forests is associated with butterfly community composition, wing optical properties and thermal biology. By combining community ecology, phylogenetic comparative analyses, multispectral imaging, field-based thermal measurements and infrared spectroscopy, the four data chapters examined these relationships across complementary spatial and functional scales. Together, the findings demonstrate that butterfly responses to tropical environments cannot be explained by a single environmental gradient or trait. Instead, species distributions were structured by substantial turnover across altitude and forest strata, while wing optical properties varied in relation to habitat, morphology and phylogenetic history. Fieldwork experiments further demonstrated that the conditions experienced by butterflies are shaped not only by ambient air temperature but also by canopy cover and solar exposure, while differences in wing nanostructure influence infrared emissivity and therefore the potential for radiative heat exchange.

6.1 Environmental effects on butterfly community composition and colour

This thesis first demonstrated that butterfly communities differed substantially in species richness, composition and phylogenetic structure across the sampled altitudinal and vertical gradients. Patterns of beta diversity indicated that differences among communities were driven primarily by species turnover rather than nestedness, suggesting that higher-altitude assemblages were not simply reduced subsets of lowland communities but contained distinct species compositions. This indicates that environmental gradients influence not only local richness, but also which species and evolutionary lineages occupy particular habitats. Vertical stratification also contributed to community differentiation, although canopy and understory assemblages generally showed greater overlap than geographically separated sites. Variation in richness between strata among sites further suggests that canopy–understory relationships depend on local ecological conditions, resource availability and habitat characteristics (Checa et al., 2014; Willig & Presley, 2016; de Souza Amorim et al., 2022; Fordyce & DeVries, 2016). As butterfly communities are often driven by species turnover, (Ki et al., 2025) this finding supports this not only across altitude, but also across smaller vertical stratification

communities. Together, these findings demonstrate that altitude and forest stratification structure butterfly communities across ecological and evolutionary dimensions, with implications for understanding how tropical assemblages may respond to environmental change.

A central finding emerging across the thesis is that the thermal and ecological environments experienced by tropical butterflies are heterogeneous, even across relatively small spatial scales. This has important implications for interpreting colouration as an adaptive trait, because the habitats occupied by butterflies differ not only in species composition but also in the environmental conditions under which their traits function (Wenda et al., 2021; Montejo-Kovacevich et al., 2016). Previous studies have demonstrated associations between butterfly colouration and thermal environments (Davis et al., 2005), including the greater prevalence of darker, larger butterflies in cooler environments (Xing et al., 2016; Günter et al., 2019) and climatic associations with visible and near-infrared reflectance across European butterfly species (Kang et al., 2021). However, these relationships are not universal. Bosi et al. (2008), for example, found no relationship between habitat temperature and wing solar absorptance across 64 lepidopteran species, while Ossola et al. (2025) found that transparent-winged Ithomiini increased in prevalence with elevation despite their lower capacity for absorbing solar radiation. These contrasting findings indicate that relationships between wing traits and thermal environments depend on the properties measured, the environmental context and the other selective pressures acting on butterfly wings.

The findings of this thesis build on this literature by demonstrating that visible reflectance varies with forest stratification, while thermal responses cannot be explained by visible colour alone. Differences in reflectance between canopy- and understory-associated butterflies may partly reflect the replacement of species with different evolutionary histories and ecological strategies across forest strata, rather than independent colour responses within individual species (Schulze et al., 2001; Fordyce & DeVries, 2016; Henriques et al., 2022). The contribution of phylogenetic relatedness to variation in reflectance supports the importance of considering evolutionary history alongside contemporary habitat associations (Henriques et al., 2022). Consequently, environmental filtering may influence which species occur in a habitat, while the optical and thermal characteristics of those

species may reflect a combination of evolutionary history and selection associated with their ecological environment. This distinction is important because differences between communities do not necessarily demonstrate that individual species have evolved particular colour traits in response to the conditions in which they are found.

The relationship between habitat and wing traits is complexified by the distinction between visible appearance and thermal function (Stuart-Fox et al., 2017; Kang et al., 2021; Berthier, 2005). Here, although visible reflectance varied with forest stratification, thermal conditions experienced by butterflies were strongly influenced by environmental exposure, with wing temperatures responding to the interaction between air temperature, altitude and forest cover. These findings are consistent with research showing that tropical forests contain substantial climatic heterogeneity associated with vegetation structure and exposure (Cómbita et al., 2022; Checa et al., 2014; Montejo-Kovacevich et al., 2020; Scheffers et al., 2017; Bennett et al., 2023), and that microclimatic conditions can influence butterfly community composition and phenology (Fordyce & DeVries, 2016; de Souza Amorim et al., 2022; Checa et al., 2014; Cómbita et al., 2022). Our findings also emphasise that air temperature alone provides an incomplete representation of the thermal conditions experienced by butterflies. Behaviour, including basking posture and wing orientation, can modify radiative heat gain and convective heat loss (Wenda et al., 2021; Kingsolver, 1988, 1987; Clench, 1966) while morphology can influence thermal responses under different environmental conditions (Bladon et al., 2020). Therefore, the thermal consequences of a particular wing colour or structure depend not only on its optical properties but also on the exposure, behaviour and morphology of the butterfly.

The analyses on infrared reflection extend this perspective by demonstrating that wing optical properties beyond the visible spectrum can differ between scale types with different visible appearances. Previous research has shown that butterfly wing microstructures influence infrared optical properties and may affect heat transfer (Krishna et al., 2020; Tsai et al., 2021l; Stuart-Fox et al., 2017). The findings presented here build on this work by examining infrared emissivity alongside visible reflectance and thermal responses in a broader ecological context. In particular, the differences in emissivity between iridescent and brown scales demonstrate that visible appearance alone does not

capture all the optical properties potentially relevant to thermal function. Structural colouration may therefore have consequences beyond visual signalling, although differences in emissivity do not, in isolation, establish how much heat an intact butterfly gains or loses under natural conditions. The thermal consequences of wing traits will also depend on their interactions with other scale properties, solar exposure and the surrounding environment.

These findings are consistent with the view that colouration can be shaped by multiple, potentially competing selective pressures, including thermoregulation, signalling and camouflage (Kemp, 2002; Kinoshita et al., 2008; Caro et al., 2017; Goldenberg et al., 2022; Ellers & Boggs, 2004; Lloyd & Nadeau, 2021). Darker pigmentation can increase solar energy absorption and may provide thermal benefits in cooler conditions, but the consequences depend on environmental exposure, signalling capabilities and behavioural thermoregulation (Kingsolver, 1987; Davis et al., 2005; Warrant & Johnsen, 2013; Günter et al., 2019; Rosa & Saastamoinen, 2020). Similarly, structural colouration can produce conspicuous visual signals through wavelength-selective reflectance, while the underlying nanostructure influences optical properties beyond the visible spectrum (Fox, 1953; Bosi et al., 2008; Chung et al., 2012; Kinoshita et al., 2008; Parnell et al., 2018). Many iridescent butterflies also possess melanised scales alongside structural colour-producing scales, creating the potential for interactions between pigmentation, structural reflectance and infrared properties (Vukusic et al., 2004; Tsai et al., 2021; Kinoshita et al., 2008; Parnell et al., 2018; Smith, 2009). These traits may consequently contribute to several functions simultaneously, rather than representing adaptations to a single environmental pressure. The distribution of different optical wing traits across habitats may additionally reflect body size, wing morphology, behaviour and phylogenetic constraints, making it important to distinguish associations between traits and habitats from direct evidence of adaptive function.

These relationships have important implications for understanding how tropical butterflies may respond to environmental change. Climate-change impacts on ectotherms are often considered primarily in terms of increasing ambient temperature (Bennett et al., 2023; Warren et al., 2021); however, the findings of this thesis suggest that changes in microclimatic conditions and habitat

structure may be equally important in determining the environments experienced by butterflies (Checa et al., 2014; Boyle et al., 2025). Deforestation and fragmentation can simplify vertical forest structure, increase exposure and alter the availability of shaded microhabitats (Tapia-Armijos et al., 2015; Warren et al., 2021). Such changes may modify thermal conditions independently of changes in regional mean temperature, with consequences for both the distribution of species and the thermal environments in which they live (Barlow et al., 2018). Given the substantial species turnover observed here, across the sampled communities, changes to forest structure and climate could affect different assemblages in different ways, rather than producing uniform changes in butterfly diversity across the landscape. In particular, the loss of structurally distinct habitats may affect species associated with particular forest strata, even where total local species richness changes relatively little.

The relationship between forest structure and wing traits may further influence how these changes affect butterfly assemblages. Canopy-associated species generally exhibited darker visible reflectance, while the emissivity analyses demonstrated that structural colouration can influence infrared optical properties. However, these patterns should not be interpreted as evidence that darker canopy colouration necessarily evolved in response to thermal conditions, because habitat associations may also reflect phylogenetic history, signalling, camouflage and other ecological processes. Instead, the findings indicate that forest structure influences both the environmental conditions under which optical traits function and the composition of the communities in which those traits occur. The consequences of environmental change may therefore emerge through interactions between altered climatic conditions, changes in microhabitat availability and differences in the traits of species occupying those habitats.

Predicting butterfly responses to future environmental change will consequently require consideration of microclimatic heterogeneity alongside broader climatic trends. Integrating measurements of solar irradiance, canopy structure, body temperature and behavioural thermoregulation with distributional, phylogenetic and optical data could provide a more comprehensive assessment of species vulnerability than approaches based solely on ambient temperature or visible colouration. Although this thesis focuses on tropical butterflies, these principles may also apply to other ectothermic

organisms inhabiting structurally complex environments, in which microclimatic variation, behaviour and organismal optical properties interact to shape thermal conditions (Val et al., 2005; Trullas et al., 2007; Scheffers et al., 2017; Chazdon & Fetcher, 1984). Overall, by integrating community composition, phylogenetic structure, visible and infrared optical properties, and thermal measurements, this thesis highlights the importance of considering butterfly traits within their broader ecological and evolutionary context. Understanding these interactions is essential not only for explaining variation in butterfly diversity and colouration across tropical habitats, but also for anticipating how these communities may respond to future environmental change.

6.2 From wing optical properties to thermal biology

The relationship between the optical and thermal chapters (Chapters 3-5) provides an important progression from wing properties to their consequences under natural environmental conditions. The multispectral analysis demonstrated strong relationships between visible and infrared reflectance and darker colouration among canopy-associated species, suggesting that visible colour may have thermal consequences. However, the field-based thermal experiments demonstrated that this relationship was more complex. Wing temperatures were strongly influenced by air temperature, altitude and canopy cover, highlighting the importance of environmental exposure in determining thermal responses.

The distinction between dead wing temperatures and the temperatures of living butterfly bodies is particularly important for interpreting these findings. In the field experiments, dead wings responded to environmental conditions, whereas live thoracic temperatures were more buffered across environmental variation. This suggests that butterflies combine passive heat gain with behavioural and physiological mechanisms that regulate body temperature (Clench, 1966; Tsai et al., 2020; Kingsolver, 1987). Previous studies have demonstrated that butterflies can actively modify heat gain through behavioural thermoregulation. For example, Kingsolver (1987) described reflectance basking in Pieris, whereby butterflies orient their wings to reflect solar radiation towards the body and demonstrated that the effects of wing melanisation on body temperature depend on wing region and basking posture. These studies illustrate how behaviour can modify, and in some cases alter, the expected thermal consequences of wing pigmentation (Kingsolver., 1987). The present findings

extend this perspective to tropical butterflies by demonstrating that thermal responses vary with environmental exposure and that measurements of dead wings, whilst having passive thermoregulation, do not necessarily predict the thermal responses of living individuals. These results also reinforce the importance of considering environmental exposure alongside intrinsic wing properties when interpreting the thermal function of colouration.

The emissivity analysis provides an additional mechanism through which wing structure may influence thermal exchange. The lower infrared emissivity of iridescent scales compared with brown scales demonstrates that scale nanostructure can alter radiative properties in ways that are not necessarily apparent from visible colouration. Structural colour may therefore be multifunctional across spectral regions, with the same scale architecture contributing to visual appearance while also influencing infrared radiation. This is consistent with previous work demonstrating thermal consequences of photonic structures in butterfly scales (Biró et al., 2003; Bosi et al., 2008; Parnell et al., 2018). This builds on research demonstrating that butterfly wing microstructures influence infrared emissivity and potentially radiative heat transfer (Krishna et al., 2020). However, previous findings also illustrate the complexity of these relationships. Bosi et al. (2008), for example, found no association between habitat temperature and solar absorptance across 64 lepidopteran species, despite finding higher average solar absorptance in iridescent specimens. Together, these studies suggest that the relationship between structural colouration and thermal function depends on the optical property measured and the environmental context, rather than following a single, predictable relationship between iridescence and temperature.

Importantly, these findings do not indicate that iridescence alone determines butterfly temperature. Rather, wing temperature emerges from interactions among environmental radiation, optical and physical properties of the wing, body morphology and behavioural and physiological regulation. The thermal experiment demonstrated the importance of habitat exposure, while the emissivity analysis demonstrated that scale structure can influence another component of radiative heat exchange. Together, these findings support a multidimensional view of butterfly thermoregulation in which

pigmentation, structural colour, scale morphology and nanostructure modify thermal exchange but do not independently determine body temperature.

6.3 Museum collections as a valuable resource

Importantly, this thesis demonstrates how natural history museum collections can be used to investigate ecological variation in optical traits across large sample of tropical butterflies. Preserved specimens provided access to 148 species spanning different ecological associations, allowing multispectral measurements to be integrated with species-level habitat distributions and phylogenetic relationships. Such taxonomic breadth would be difficult to achieve through field sampling alone, particularly for species that are rare, seasonally restricted, difficult to capture or geographically separated. Museum collections therefore provide an opportunity to extend ecological analyses beyond the spatial and temporal constraints of contemporary field sampling (Castillo-Figueroa, 2018; Newbold, 2010).

When combined with collection records, geographical information and phylogenetic data, museum specimens can provide a powerful comparative framework for investigating how traits vary among species and environments (Pyke & Ehrlich, 2010; Meineke & Davies, 2018; MacLean et al., 2019). This creates opportunities to examine questions extending beyond colour description, including the evolution of structural colour, relationships between optical traits and habitat, and potential associations between morphology and thermal ecology.

However, museum-based measurements cannot capture behavioural thermoregulation, microhabitat selection or dynamic exposure to solar radiation, and optical properties may also be affected by specimen age, preservation history and scale condition. Museum measurements are therefore most appropriately interpreted as measurements of intrinsic wing properties rather than direct measurements of the thermal environment experienced by living butterflies. The combination of museum and field approaches used in this thesis illustrates their complementary value: museum specimens provide the taxonomic breadth required to identify broad comparative patterns, while field experiments allow those patterns to be evaluated within the environmental and behavioural context in

which they function. Continued digitisation and integration of natural history collections with ecological, climatic and phylogenetic datasets could further expand their value for investigating trait variation and responses to environmental change (Beaman & Cellinese, 2012).

6.4 Thesis Conclusions

Together, this thesis provides an integrated understanding of how environmental heterogeneity shapes tropical butterfly diversity, wing optical properties and thermal ecology. By combining community ecology, phylogenetic comparative analyses, multispectral imaging, museum specimens and field-based thermal measurements, this research demonstrates that butterfly responses to tropical environments emerge from interactions among broad-scale gradients, such as altitude and geographic separation, and fine-scale variation in habitat structure and environmental exposure.

At the community level, butterfly assemblages were strongly differentiated across both altitudinal and vertical forest gradients, with species turnover contributing substantially to differences among communities. Phylogenetic clustering at some higher-elevation sites further indicated that environmental filtering may influence which lineages persist under different environmental conditions. These patterns demonstrate that tropical butterfly diversity is maintained not only through high local species richness, but also through the spatial replacement of species and evolutionary lineages across heterogeneous environments. Wing optical properties similarly varied across environmental and biological contexts. Visible reflectance was associated with forest stratification, while phylogenetic history and body size also contributed to variation in optical traits. The relationship between visible and infrared reflectance indicated that pigmentation can influence passive thermal properties, whereas differences in iridescent and brown scales demonstrated that structural colour and nanostructure can alter infrared properties independently of visible appearance. These findings emphasise that butterfly colouration is multifunctional, reflecting interacting pressures associated with thermoregulation, signalling, camouflage, morphology and evolutionary history. However, the fieldwork experiments demonstrated that wing colour alone does not determine thermal state. Wing temperatures were strongly influenced by environmental conditions, particularly air

temperature, altitude and habitat exposure, while live thoracic temperatures showed evidence of additional regulation. Together with the emissivity results, these findings demonstrate that butterfly thermal biology emerges from interactions between environmental exposure, wing optical and structural properties, morphology, and behavioural and physiological regulation.

More broadly, this thesis demonstrates the value of integrating ecological, evolutionary and physiological approaches to understand how tropical insects respond to heterogeneous environments. Museum specimens provided the taxonomic breadth necessary to identify comparative patterns in optical traits, while field experiments captured the environmental and thermal context in which those traits function. This combination provides a framework for linking species distributions and community assembly with the evolution and function of organismal traits.

As tropical forests continue to experience climate change and habitat modification, understanding these interactions will be increasingly important. Changes in temperature, forest structure and exposure to solar radiation may alter both the composition of butterfly communities and the thermal environments in which species operate. Future research combining long-term ecological monitoring with direct measurements of microclimate, behavioural thermoregulation, wing nanostructure and genomic variation will help clarify the mechanisms underlying species' resilience and vulnerability.

Ultimately, this thesis demonstrates that understanding tropical butterfly responses to environmental change requires moving beyond single gradients or traits. The diversity, distribution and thermal biology of butterflies are shaped by the interaction between their evolutionary history, physical traits and the heterogeneous environments they inhabit.

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