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Parallel shifts in flight-height associated with altitude across incipient *Heliconius* species

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Abstract

Vertical gradients in microclimate, resource availability, and interspecific interactions are thought to underly stratification patterns in tropical insect communities. However, only a few studies have explored the adaptive significance of vertical space use during the early stages of reproductive isolation. We analysed flight-height variation across speciation events in *Heliconius* butterflies, representing parallel colonizations of high-altitude forest. We measured flight-height in wild *H. erato venus* and *H. chestertonii*, parapatric lowland and mountain specialists, respectively, and found that *H. chestertonii* consistently flies at a lower height. By comparing our data to previously published results for the ecologically equivalent *H. e. cyrbia* (lowland) and *H. himera* (high altitude), we found that the species flying closest to the ground are those that recently colonized high-altitude forests. We show that these repeated trends largely result from shared patterns of ecological selection producing parallel trait-shifts in *H. himera* and *H. chestertonii*. Although our results imply a signature of local adaptation, we did not find an association between resource distribution and flight-height in *H. e. venus* and *H. chestertonii*. We discuss how this pattern may be explained by variations in forest structure and microclimate. Overall, our findings underscore the importance of behavioural adjustments during early divergence mediated by altitude-shifts.

Resumen

Se cree que los gradientes verticales microclimáticos, de disponibilidad de recursos y de interacciones interespecíficas, subyacen a los patrones de estratificación en las comunidades de insectos tropicales. Sin embargo, solo unos pocos estudios han explorado la importancia adaptativa del uso del espacio vertical durante los primeros estados del aislamiento reproductivo. Analizamos la variación en la altura de vuelo en eventos de especiacion en mariposas Heliconius que representan colonizaciones paralelas de bosques de alta montaña. Medimos la altura de vuelo de individuos silvestres de *H. erato venus* y *H. chestertonii*, que son especialistas parapátricos de tierra baja y alta montaña respectivamente y encontramos que *H. chestertonii*, de manera consistente, vuela a una altura menor. Comparando nuestros datos con los publicados previamente para los equivalentes ecológicos *H. e. cyrbia* (tierra baja) y *H. himera* (alta montaña), encontramos que las especies que vuelan más cerca al suelo son aquellas que recientemente colonizaron bosques de alta montaña. Demostramos que estas tendencias repetidas son en gran parte el resultado de patrones compartidos de selección ecológica que producen cambios paralelos en los rasgos de *H. himera* y *H. chestertonii*. Si bien nuestros resultados sugieren indicios de adaptación local, no encontramos evidencia de una asociación entre la distribución de recursos y la altura de vuelo de *H. e. venus* y *H. chestertonii*. Discutimos como esto puede ser explicado por variaciones en la estructura del bosque y el microclima. En general, nuestros hallazgos resaltan la importancia de los ajustes comportamentales al comienzo de la divergencia mediada por cambios de altura.

Keywords: Heliconiini, local adaptation, micro-habitat, parallel evolution

Introduction

The steep environmental gradients that characterize the upper and lower vegetation layers of tropical forests are a key factor determining species diversity and abundance (Basham et al., 2022; Basset et al., 2001, 2015; Jucker et al., 2020; Scheffers et al., 2013). In arthropods, particular species are consistently found in some vertical strata while being less common in others due to variations in abiotic conditions, behaviour, and the distribution of resources (Basset et al., 2003; Wardhaugh, 2014). For example, fruit-feeding and nectar-feeding butterflies, respectively, decrease and increase in abundance towards the canopy, ostensibly tracking their food sources (De Vries, 1988; Schulze, 1998; Schulze et al., 2001). This has broad implications for the structure of communities, with consequences for wider trophic interactions (Elias et al., 2008; Willmott et al., 2017).

Comparatively less is known about vertical segregation between closely related populations. Only a handful of studies have examined divergence in the use of vertical

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space at this level, but these provide a range of evidence implicating flight differences in early divergence. For instance, *Heliconius* flying in separate vertical strata have different wing morphologies (Mena et al., 2020). Similarly, the Nymphalid *Archaeoprepona demophon* includes coexisting canopy and undercanopy populations with independent demographic dynamics and low levels of gene flow (Nice et al., 2019). Differences in resource use and larval behaviour have also been linked to segregation between *Colobura annulata* and *C. dirce*, which were previously considered a single species but are now separated based on differences in vertical space use: *C. annulata* are gregarious and feed on mature, canopy *Cecropia* leaves, while *C. dirce* are solitary and consume undercanopy saplings (Willmott et al., 2001). These examples suggest a role for vertical space in local adaptation, but the underlying mechanisms remain unclear.

Recently, heritable flight-height differences were demonstrated between the species *H. e. cyrbia* and *H. himera* (Dell'Aglio et al., 2022), which are distributed along a gradient from wet, low-altitude forest to dry, high-altitude forest, with a narrow zone of overlap where they hybridize (Jiggins et al., 1996). Flight-height correlates with the distribution of resources in their habitats such that *H. himera* flies lower than *H. e. cyrbia*, tracking the reduced height of hostplants and flowers at high altitude (Dell'Aglio et al., 2022). This pattern may result from divergent ecological selection maximizing hostplant encounter rates and foraging efficiency across forests with different structures, potentially contributing, alongside other factors (Davison et al., 1999; McMillan et al., 1997; Merrill et al., 2014; Montgomery and Merrill, 2017; Rivas-Sánchez et al., 2023), to the evolution of reproductive isolation.

In the western slope of the Colombian Cordillera Occidental, *H. e. venus* and *H. chestertonii* are distributed along a similar cline. *H. e. venus* occupies wet forests below 1,500 m and is replaced eastwards, at higher altitudes, by its close relative *H. chestertonii*, which is adapted to drier and colder scrub forest (Arias et al., 2008; Brower, 1996; Rivas-Sánchez et al., 2023). *Heliconius chestertonii* clusters independently from *H. e. venus* in genetic analysis, despite some degree of hybridization, akin to the relationship between *H. himera* and its three parapatric lowland *H. erato* populations (*H. e. cyrbia*, *H. e. favorinus*, and *H. e. emma*) (Van Belleghem et al., 2021). Importantly, *H. chestertonii* and *H. himera* are also genetically distinct, with no documented hybridization or gene flow, and form paraphyletic lineages with respect to lowland *erato* populations (Van Belleghem et al., 2021). Thus, *H. chestertonii* and *H. himera* can be viewed as two independently derived Andean populations (Rosser et al., 2012) that are ecologically equivalent (Rivas-Sánchez et al., 2023). We leveraged these replicate colonizations of the high Andes to explore the relationship between vertical space use and divergence among parapatric populations at the early stages of reproductive isolation. In particular, we tested whether *H. e. venus* and *H. chestertonii* fly at different heights and if this correlates with the location of larval and adult-stage resources in their respective habitats. Assuming that flight-height patterns constitute adaptive traits shaped by ecological selection, we predicted that flight-height variation between *H. e. venus* and *H. chestertonii* would mirror that between the ecologically comparable *H. e. cyrbia* and *H. himera*.

Materials and methods

Habitat characterization

We investigated *Heliconius* butterflies in sites located in the habitats of *H. e. venus* (Buenaventura, N03°50′0.04″ W77°15′45.1″ and La Barra, N03°57.558420 W 77°22.705080) and *H. chestertonii* (Montañitas N03°40′36.3″ W76°31′19.4″ and El Saladito N03°29′11.5″ W76°36′39.8″) in January 2021. These sites are away from the contact zone between the two species and thus represent allopatric conditions. We characterized the local forest structure by measuring the trunk diameter at breast height (m) and the average distance (m) between neighbouring trees of common tree species along 15–20 m sections of the trails used for collecting butterflies. Following Dell'Aglio et al. (2022), the plants were assigned to a forest layer depending on (a) whether the tree crown appeared mature and was not shaded by surrounding trees (canopy), (b) the crown was not mature and was positioned below a canopy tree (undercanopy), or (c) the plant was a herbaceous or a young seedling (shrubs). To estimate the height (m) of plants from each category in these sections of trail, we used two approaches. For the tallest trees, we measured the angle of elevation to the treetop with a clinometer. The tangent of this angle was then multiplied by the distance to the tree from the point of observation to estimate the height. Shorter trees were measured using measuring tapes.

Vertical distribution of resources

We explored whether the distribution of adult and larval resources differed between habitats. To do this, we measured the height (m) of all the *Passiflora* (Passifloraceae) hostplants that were visually identified along the trails used to collect butterflies. These plants were subsequently identified to the species level (Pérez, 2007; Ulmer & MacDougal, 2004) and filtered to include only *Passiflora* species known to be used by the *erato* complex (Jiggins, 2017; Robinson et al., 2010), to which our focal species belong. We also measured the height (m) of the flowering plants that butterflies were observed feeding on at least once. Note that these methods differ slightly from Dell'Aglio et al. (2022), where plants were identified by following butterflies until they landed on a flower or hostplant. Due to the trail and forest structure, this approach was not possible here.

Patterns of flight-height divergence

In our study, flight-height is defined as the length of the line perpendicular to the ground level from the point where individuals were first observed, as estimated with a measuring tape. Each measured individual (*H. e. venus* $n = 33$ and *H. chestertonii* $n = 39$) was stored, ensuring no repeated observations. To quantify levels of parallelism in flight-height between butterflies from independent, ecologically equivalent pairs, we included flight-height measures of *H. e. cyrbia* ($n = 78$) and *H. himera* ($n = 86$) in our dataset, available in Dell'Aglio et al. (2022).

Statistical analyses

To test for significant differences in the diameter at the breast height, the average distance between neighbouring trees, and the height of different forest layers between habitats, we used Wilcoxon rank sum tests implemented in R (R Core Team, 2020). We also modelled variation in the height of hostplants and flowers separately using linear mixed-effect models (implemented in *lme4*; Bates et al., 2014) with "habitat" as a fixed factor and "site" as a random effect. This was followed by Wald χ^2 tests to test for a significant effect of the "habitat" on each response variable (implemented with the *Anova* function in the package *CAR*; Fox and Weisberg, 2018). To analyse flight in *H. e. venus* and *H. chestertonii*, we used linear-mixed models where variation in flight-height was explained by the fixed factors "species" and "sex," as well as interactions and the random factor "site." We subsequently used the *drop1* function (base R) to remove model terms that did not contribute to model fit, as measured using likelihood tests between alternative models.

Finally, to test for parallel shifts in flight-height across independent divergence events, we used a variance partitioning approach (Langerhans & DeWitt, 2004) combining data on *H. e. venus*, *H. chestertonii*, *H. e. cyrbia*, and *H. himera*. This method assumes populations that have been independently exposed to derived conditions. Our model included the main factors "habitat" (similarity of selection regimes across independent divergence events) and "locality" (variation in flight-height that responds to the unique properties of each divergence), as well as their interaction (inconsistency in magnitude and/or direction of divergence patterns of the two pairs of species). We assessed the significance of each of these terms via likelihood (*drop* 1 function) and Wald χ^2 tests between alternative models. We then estimated semi-partial R^2 coefficients (*r2beta* function, Kenward–Roger method in the R package r2glmm; Jaeger, 2017) in our final model, providing a rough approximation of the proportion of variance explained by each term.

Results

The habitats of *H. e. venus* and *H. chestertonii* broadly differed (Figure 1A–C). In particular, the trunk diameter at the breast height (W = 32, p = .022) and the distance between neighbouring trees (W = 15, p = .001) were both significantly higher in the habitat of *H. e. venus*. The height of the canopy and shrub forest layers were not significantly different between the two forest types, but the undercanopy was significantly shorter in the habitat of *H. chestertonii* (W = 30, p = .006). We did not identify between-habitat differences in the vertical distribution of hostplants (n = 37, χ^2_1 = 0.394; p = .529) or flower resources (n = 107, χ^2_1 = 0.009; p = 0.921) (Figure 2A and B).

On average, *H. chestertonii* flew ~0.5 m lower than *H. e. venus* (n = 74, χ^2_1 = 8.860; p = .002; Figure 2C), detected

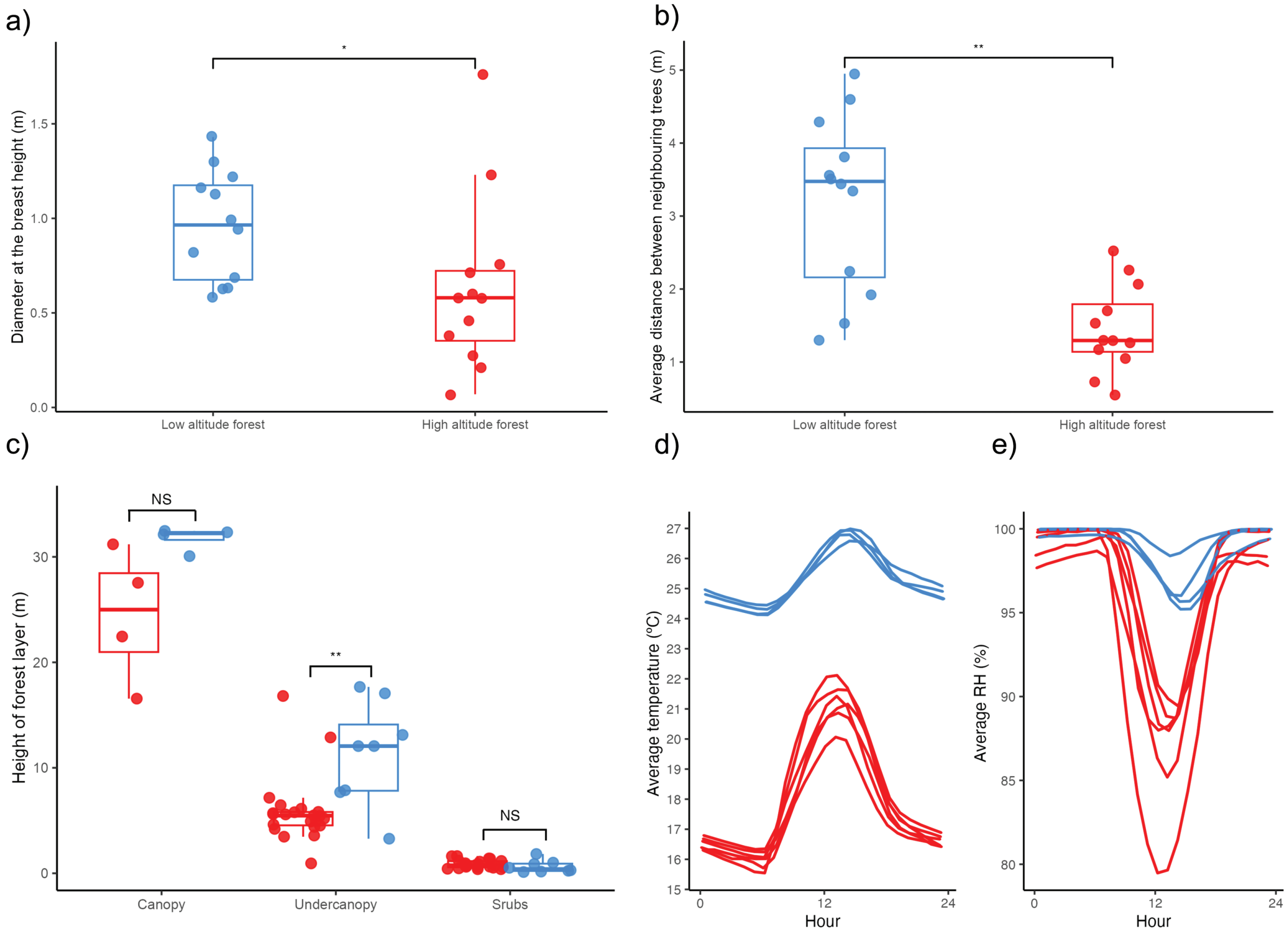

Figure 1. Characteristics of low-altitude and high-altitude habitats. Comparison of (A) diameter at the breast height (DBH); (B) average distance between neighbouring trees (ADNT); (C) canopy, undercanopy, and shrub heights; (D) temperature; and (E) relative humidity throughout the day. In (A, B), box plots show median and upper and lower quartiles, each point is an individual. Significance levels (Wilcoxon test): NS, not significant. *$p < .05$. **$p < .01$. In (D, E), lines represent average hourly microclimate recorded by individual dataloggers in low- or high-altitude forest (created from data retrieved from Rivas-Sánchez et al., 2023).

in models with "species" as the only main factor. Moreover, likelihood-ratio comparisons between models combining data from *H. e. venus*, *H. chestertonii*, *H. e. cyrbia*, and *H. himera* showed that the "habitat" ($n = 240$, $\chi^2_1 = 21.725$; $p < .001$) and "locality" ($n = 240$, $\chi^2_1 = 3.894$; $p < .048$) effects were both significant, but their interaction was not, and "habitat" explained over 50% of the variance in flight-height (Table 1). This suggests a close correspondence between the patterns of flight-height divergence between *H. e. venus*–*H. chestertonii* and *H. e. cyrbia*–*H. himera*, despite the larger magnitude of mean difference (~1.5 m) in *H. e. cyrbia*–*H. himera* (Figure 2D).

Discussion

We leveraged phylogenetically independent colonizations of high-altitude forest, analysed as ecologically equivalent pairs of local, parapatric populations in Colombia and Ecuador to explore local adaptation in the context of altitude-shifts. We demonstrate that the mountain specialist *H. chestertonii* flies at a lower height compared with the parapatric, low-altitude *H. e. venus*, with which it forms a narrow hybrid zone, mirroring a pattern formerly reported in *H. himera* and *H. e. cyrbia* (Dell'Aglio et al., 2022). This strongly implies a role for related sources of divergent selection driving local adaptation in flight-height across habitat types.

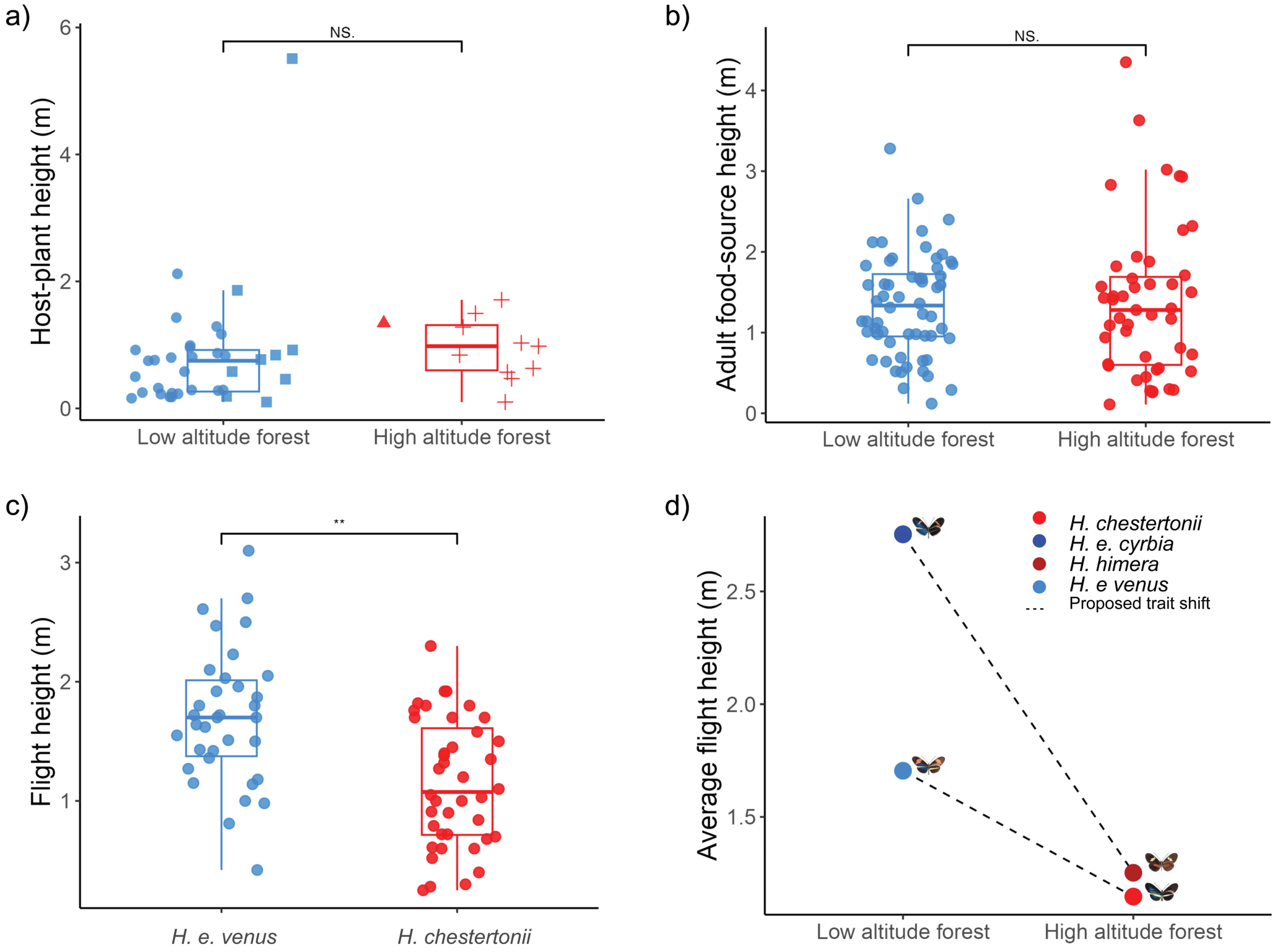

Figure 2. Comparisons in resource distribution and flying-heights in *Heliconius* species. (A) Hostplants, (B) adult-food sources, (C) flight-heights, and (D) parallelism between different clines. For (A), circles are *Passiflora auriculata*, squares are *P. micropetala*, pluses are *P. suberosa* and triangles *P. biflora*. Box plots show median and upper and lower quartiles. Significance levels (A–B) (Wilcoxon test): NS, not significant. Significance level in (C) (Wald χ^2 test): $**p > .01$.

Table 1. Semi-partial R^2 measures of terms in model explaining variation in flight-height.

Semi-partial R^2 measures (95% CI)				
Model	**Factor**	**Semi-partial R^2**	**Upper CI**	**Lower CI**
Flight-height~Habitat + Locality	Habitat	0.622	0.843	0.325
	Locality	0.238	0.639	0.004
	Model	0.636	0.851	0.371

Adaptation can be inferred when parallel phenotypic differentiation occurs across replicate populations in similar environments (Langerhans & DeWitt, 2004; Losos et al., 1998; Schluter, 2000). The derived species *H. chestertonii* and *H. himera* independently colonized similar mountainous habitats, as demonstrated from genomic data isolating them in two distinct clusters separated from adjacent *H. erato* populations despite incomplete reproductive isolation (Van Belleghem et al., 2021). We measured flight-height in wild individuals, thus not explicitly ruling out the possibility of environmentally induced responses underlying trait variation. However, *H. e. cyrbia* and *H. himera* retain their differences in flight-height in common garden conditions (Dell'Aglio et al., 2022), and this may also be the case for the closely related *H. chestertonii* and *H. e. venus*, opening the trait to selection across the Colombian cline. Regardless, our tests for parallelism suggest these independent shifts in flying behaviour are largely driven by shared sources of divergent selection, rather than stochastic evolutionary processes (e.g., genetic drift) or unique selection regimes (Langerhans & DeWitt, 2004; Losos et al., 1998; Schluter, 2009) and thus potentially indicate repeated local adaptation (e.g., Colosimo et al., 2005; Cresko et al., 2004; Gillespie et al., 2018; Johnson et al., 2000; Projecto-Garcia et al., 2013).

In contrast to *H. e. cyrbia* and *H. himera* (Dell'Aglio et al., 2022), we detected no associations between flight-height variation in *H. e. venus* and *H. chestertonii* and the vertical distribution of plant resources. In other species, such correlations are interpreted as an indication that butterflies fly at heights that optimize encounter rates with food sources (Beccaloni, 1997; De Vries, 1988; DeVries et al., 2010; Schulze et al., 2001). *Passiflora auriculata* was the only suitable hostplant in *H. e. venus* habitat, whereas we found *P. biflora* and *P. suberosa* in *H. chestertonii* habitat. These three *Passiflora* belong to the *Decaloba* subgenus and grow to modest dimensions (Ulmer & MacDougal, 2004), likely explaining their similar heights. This lack of differentiation conflicts with the hypothesis that flight-height is selected to maximize foraging and oviposition opportunities. Interestingly, in the low-altitude, *H. e. venus* site La Barra, we found plants of a fourth species, *P. micropetala*, which grew up to 6 m tall. We did not include this species in our analyses, as it is unknown if *H. erato* uses it (although its inclusion does not quantitatively affect our results given the small sample size of recorded plants). Nevertheless, *P. micropetala* is a near relative to *P. biflora* (Ulmer & MacDougal, 2004), and *Heliconius erato* are known to seasonally switch hosts when their preferred hostplant is depleted (Benson, 1978; Kerpel & Moreira, 2005; Ramos et al., 2012; Rodrigues & Moreira, 2004). Regardless, the presence of these plants in La Barra resonates with the idea that the lowland forest undercanopy offers support for taller *Passiflora* (Dell'Aglio et al., 2022; Pérez, 2007). Further studies could confirm whether *H. e. venus* uses *P. micropetala*, which may relate to its higher flight-height compared with *H. chestertonii*.

As reported in previous studies (Giraldo-Pamplona et al., 2012), we found that the structure of high- and low-altitude forests in the West of Colombia differs such that the former, occupied by *H. chestertonii*, has generally shorter trees at higher densities. This habitat is also colder and drier, with stronger microclimatic fluctuations (Figure 1D and E). These differences mirror those between the habitats of *H. e. cyrbia* and *H. himera* (Dell'Aglio et al., 2022) and may result in shared sources of divergent selection across both pairs of taxa. For instance, oviposition behaviour in butterflies is affected by microclimatic conditions (Eilers et al., 2013; Turlure & Van Dyck, 2009). Using dummy models, Merckx et al. (2008) have also showed that *Pararge aegeria* warms up faster when ambient temperatures are higher, experiencing less convective cooling at lower heights (0.9m difference) in forests with a more sheltered structure. In insects, pupation-height is partly driven by desiccation risk, which decreases close to the ground (Rodriguez et al., 1990; Schnebel & Grossfield, 1986). This could influence the flight behaviour of males of pupal-mating *Heliconius*. It is therefore plausible that *H. e. venus* and *H. chestertonii* are flight-height adjusted to compensate for habitat differences influencing microclimate-related traits.

In conclusion, we showed that two derived mountain specialist *Heliconius* have independently evolved flight behaviours via trait-shifts similar in trajectory, suggesting local adaptations in forests at different elevations. In Colombia, we do not find evidence to confirm that this pattern is driven by differences in the vertical distribution of resources. We suggest that these parallel differences may instead relate to the effects of microclimatic conditions, variable between forest types. These results add to a limited list of studies exploring potential mechanisms underlying differences in vertical space use between closely related insect taxa.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

Supplementary information and raw data can be found in Supplementary Tables S1–S4 and annotated R scripts. Files are available in the Dryad Digital Repository (https://doi.org/10.5061/dryad.6hdr7sr6h).

Author contributions

David F Rivas-Sánchez (Data curation [lead], Formal analysis [lead], Investigation [equal], Writing—original draft [lead], Writing—review & editing [equal]), Carlos H Gantiva-Q (Investigation [equal], Writing—review & editing), Carolina Pardo-Díaz (Project administration [equal], Resources [equal], Supervision [equal], Writing—review & editing), Camilo Salazar (Project administration [equal], Resources [equal], Supervision [equal], Writing—review & editing), Stephen Montgomery (Conceptualization [equal], Funding acquisition [equal], Methodology [equal], Validation [equal], Writing—original draft-Supporting, Writing—review & editing), Richard Merrill (Conceptualization [equal], Funding acquisition [equal], Methodology [equal], Validation [equal], Writing—original draft [supporting], Writing—review & editing)

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Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

Fieldwork was conducted under permit no. 530 issued by the Autoridad Nacional de Licencias Ambientales of Colombia (ANLA). Our study complied with the relevant guidelines of insect research.

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