Title:
Divergence in brain composition during the early stages of ecological specialisation in Heliconius butterflies

Authors:
Stephen H. Montgomery1,2*
Richard M. Merrill2

Affiliations:
1 Dept. Genetics, Evolution & Environment, University College London, Gower Street, London, UK, WC1E 6BT
2 Dept. Zoology, University of Cambridge, Downing Street, Cambridge, UK, CB2 3EJ

* Corresponding author
Email: Stephen.Montgomery@cantab.net
Tel: +44 (1)22336678
Fax: N/A

Short running title: Brain evolution and habitat divergence
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Abstract:
During speciation across ecological gradients diverging populations are exposed to contrasting sensory and spatial information that present new behavioural and perceptive challenges. These challenges may be met by heritable or environmentally-induced changes in brain function which mediate behaviour. However, few studies have investigated patterns of neural divergence at the early stages of speciation, inhibiting our understanding of the relative importance of these processes. Here, we provide a novel case study. The incipient species pair, Heliconius erato and H. himera are parapatric across an environmental and altitudinal gradient. Despite ongoing gene flow, these species have divergent ecological, behavioural and physiological traits. We demonstrate that these taxa also differ significantly in brain composition, in particular in the relative levels of investment in structures that process sensory information. These differences are not explained solely by environmentally-induced plasticity, but reflect heritable, non-allometric shifts in brain structure. We suggest these differences reflect divergence to meet the demands of contrasting sensory ecologies. This conclusion would support the hypothesis that the evolution of brain structure and function play an important role in facilitating the emergence of ecologically distinct species.
Introduction

Local adaptation following the colonisation of novel environments can facilitate the origin of new species (Darwin, 1859; Rice, 1987; Nosil, 2012). During the early stages of this process, diverging populations are exposed to contrasting sensory information, and changes in the spatial distribution of resources. This can present new behavioural and perceptive challenges. These can be met by changes in brain function, often reflected in differential investment in brain components (Striedter, 2005). Recent studies considering variation within species highlight the potential for neural plasticity to optimise brain composition to local conditions (Eifert et al., 2011; Gonda et al., 2011; Snell-Rood, 2013). Plasticity can also facilitate speciation by permitting survival in novel environments, and prolonged exposure to associated selection pressures (Price et al., 2003; Pfennig et al., 2010). However, developmental plasticity has associated functional and energetic costs (Snell-Rood et al., 2009; Snell-Rood, 2013), and between species analyses demonstrate that heritable changes in brain composition are ultimately required to meet the demands of a species’ ecology (e.g. Poulson & White, 1969; de Winter & Oxnard, 2001; Catania, 2005; Jeffery, 2005). Little is known about the role of brain evolution and plasticity at the intersection of these evolutionary scales when new species emerge from locally specialised populations.

Here, we investigate the role of heritable divergence and plasticity in brain composition during the early stages of ecological speciation in Heliconius butterflies. Speciation in Heliconius often involves adaptive ecological divergence (Merrill et al., 2015), and a number of extant taxon-pairs provide ‘snap-shots’ of this process at different stages of completion (Mallet et al., 2007; Merrill et al., 2011). H. himera and H. erato cyrbia are distributed across an environmental gradient, and reflect the transition between polymorphic races and distinct species. H. himera is an incipient species emerging from within the H. erato clade (Supple et al., 2015). Unlike low altitude races of H. erato, which are typically found in large-leaved secondary wet forest, H. himera is endemic to high altitude dry forest in the western border of Ecuador and Peru (Descimon & Mast de Maeght, 1983). This parapatric distribution exposes individuals to different environmental conditions, including different light environments, average rainfall and daily temperature range (Jiggins et al., 1996; McMillan et al., 1997; Davison et al., 1999). These contrasting abiotic conditions in turn shape differences in forest and foliage type and density, the distribution of
resources, and the ecological communities and predators individuals experience (Jiggins et al., 1996). This biotic change in habitat type, between wet and dry forest, is thought to be the primary factor isolating these species, rather than altitude per se or genetic incompatibilities (Jiggins et al., 1996; McMillan et al., 1997).

Adaptation to the contrasting conditions of wet and dry forest has therefore played a central role in driving and maintaining divergence in these butterflies (Jiggins et al., 1996, 1997; McMillan et al., 1997; Davison et al., 1999). Shifts in coloration likely reflect adaptive optimisation of warning signaling to different communities of predators in either habitat, illustrating the biotic discontinuity between these forest types. In populations of both H. erato and H. himera, frequency-dependent predation of rare Heliconius warning patterns (Mallet, 1989; Merrill et al., 2012; Chouteau et al., 2016), is augmented by assortative mating (McMillan et al., 1997; Mallet et al., 1998; Merrill et al., 2014). Their contrasting environments have also driven number of phenotypic changes beyond color pattern, including changes in daily activity pattern and life history traits (Davison et al., 1999). Both migrant and hybrid individuals are thought to suffer fitness costs when poorly matched to their environment, at least in part due to behavioural and/or physiological divergence, suggesting they are under strong, divergent ecological selection pressures (McMillan et al., 1997). This ecological specialisation persists despite ongoing gene flow across a narrow contact zone, in which 5-10% of individuals are of hybrid origin (Jiggins et al., 1996, 1997). This high rate of hybridisation emphasises the recent origin of H. himera. For example, the frequency of hybrids observed between the sympatric species pair H. melpomene and H. cydno, which diverged ~1 million years ago (Kozak et al., 2015), is less than 0.1% (Mallet et al., 1988).

Behavioural and sensory adaptations that allow populations to colonize and adapt to new habitats are likely mediated by the evolution of brain function, which in turn may often involve changes in brain structure. This could affect peripheral brain regions causing changes in sensitivity or the way salient information is extracted from sensory information, or higher order brain regions that integrate sensory cues and coordinate the behavioural response. Here, we take advantage of the recent ecological divergence of H. himera and H. erato to provide an initial test of this hypothesis. We demonstrate that despite this recent origin, H. himera and H. erato have robust differences in the relative size of multiple brain components. These species differences are not solely explained by environmentally-induced developmental
plasticity, suggesting changes in brain structure and function may have contributed to
the emergence of *H. himera*.

**Materials and methods**

**Animals**

Wild *H. himera* and *H. erato* were collected from the forests around Vilcabamba and
Balsas Canton, respectively, in Southern Ecuador (Figure 1). These populations lie
either side of a narrow hybrid zone, occupy divergent habitats and show divergent
behavioural and life history traits (Jiggins *et al.*, 1996; McMillan *et al.*, 1997; Davison
*et al.*, 1999; Merrill *et al.*, 2014). Eight wild male and eight wild female individuals of
each species were included in the analyses. Individuals were collected using hand nets
and kept alive in glassine envelopes until brain tissue could be fixed, typically within 5
hours of collection. Body length (maximum anterior-posterior distance [mm]),
wingspan (maximum distance [mm] between the apex of the forewings) and body
mass [mg]) were measured for all individuals. We found no evidence for any species
difference in any of these body size measurements (see Supporting Information A;
Figure S1).

Insectary-reared individuals were bred from wild-caught females in common
garden conditions. Adults were kept under standard conditions in outbred stock cages
(c. 1 × 2 × 2 m) of mixed sex and equal densities. Stock cages contained a minimum
of 10 females. Larvae were reared on a common host plant (*Passiflora biflora*). *H.
himera* and *H. erato* have no known differences in host plant preference; in the wild
both species utilise *P. rubra* and *P. punctata* (Jiggins *et al.*, 1996). *P. biflora* is not
native to either habitat, but is more closely related to *P. punctata* than *P. rubra*
(Yockteng and Nadot 2004), host-plant divergence plays no role in the speciation
event between *H. erato* and *H. himera* and both species readily lay on multiple
*Passiflora* including *P. biflora* and larvae show no differences in survival (Jiggins *et
al*. 1997b). We therefore suggest it is extremely unlikely any species differences
reflect contrasting responses to the *P. biflora*. Insectary-reared individuals showed no
significant difference to wild caught individuals in body length or wingspan
suggesting they suffer no significant dietary stress as larvae on *P. biflora* (all
comparisons \(p>0.05\)). Eggs were collected from the host plants on a daily basis over
an 8-week period, and separated until hatching. Individual larvae were then raised on
P. biflora new growth shoots in a climate-controlled room (at 28°C, 100% humidity and a 12 hour light dark cycle). Pupae were maintained under the same conditions until eclosion. After eclosion, adults were aged for 10-14 days in mixed cages, at which point both sexes are sexually and behaviourally mature. Five individuals of each sex were sampled for both species.

Immunocytochemistry and imaging

Brains were fixed in-situ using a Zinc-Formaldehyde solution, following a published protocol (Ott, 2008). Further methodological details and anatomical descriptions of the Heliconius brain are available in Montgomery et al., (2015). Briefly, brain structure was revealed using immunofluorescence staining against a vesicle-associated protein at presynaptic sites, synapsin (anti-SYNORF1; obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA; RRID: AB_2315424) and Cy2-conjugated affinity-purified polyclonal goat anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), obtained from Stratech Scientific Ltd., Newmarket, Suffolk, UK (Jackson ImmunoResearch Cat No. 115-225-146, RRID: AB_2307343).

All imaging was performed on a confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystem, Mannheim, Germany) using a 10× dry objective with a numerical aperture of 0.4 (Leica Material No. 11506511), a mechanical z-step of 2 μm and an x-y resolution of 512 × 512 pixels. The z-dimension was scaled 1.52× to correct the artifactual shortening (Montgomery et al., 2015). We assigned image regions to brain components, or neuropils, using the Amira 5.5 labelfield module and defining outlines based on the brightness of the synapsin immunofluorescence. We reconstructed total central brain volume (CBR), six paired neuropils in the optic lobes, six paired and one unpaired neuropils in the central brain (CBR), and measured their volume using the measure statistics module. These neuropils include the major visual and olfactory neuropils, as well as the mushroom bodies, structures linked to learning and memory (Zars, 2000), and components of the central complex, a multimodal integration and action selection centre (Strauss, 2002; Bender et al., 2010; Pfeiffer & Homberg, 2014). The total volume of segmented structures in the CBR was subtracted from total CBR volume to obtain a measure of unsegmented CBR (rCBR). Due to the
lack of volumetric asymmetry in *Heliconius* neuropils (Montgomery *et al.*, 2015) we measured the volume of paired neuropils from one hemisphere, chosen at random unless one hemisphere was damaged, and multiplied the measured volume by two. All volumes were log$_{10}$-transformed before data analysis.

**Statistical analyses**

Principal Component Analyses (PCA) were performed using all segmented structures and rCBR. Significant PC were defined as those with Eigenvalues >1. Species differences in PC values were analysed using an ANOVA, including species and sex as binary cofactors, in R (R Development Team, 2008). We complemented this analysis with a Discriment Function Analysis (DFA) to test how reliably individuals can be assigned to their respective groups on the basis of their volumetric differences in neuropils. In this analysis, Wilks’ $\lambda$ provides a measure of the proportion of total variance not explained by group differences, and the $\chi^2$ statistic provides a test for significant group differences. Multivariate analyses were performed in SPSS v. 22 (SPSS Inc., Chicago, IL).

We further explored whether the scaling relationships between each component and a measure of overall brain size were conserved across *H. himera* and *H. erato* using major axis regressions in SMATR v.3.4-3 (Warton *et al.*, 2012) following Ott and Rogers (2010). rCBR was used as the measure of overall brain size in the main text as it allows comparisons of individual neuropil that are fully independent of one another; but see Supporting Information A for alternative measures. Using the standard allometric scaling relationship: $\log y = \beta \log x + \alpha$, we performed tests for significant shifts in the allometric slope ($\beta$) between the species. This was followed by two further tests which assume a common slope: 1) for differences in $\alpha$ that suggest discrete ‘grade-shifts’ in the relationship between two variables, 2) for major axis-shifts along a common slope. Conserved scaling relationships are typically interpreted as indicating the presence of some constraint that results in covariance between variables. This constraint may arise from shared developmental mechanisms (or pleiotropy), or be due to selective covariance to maintain a constant level of functional integration (Armbruster & Schwaegerle, 1996). Under this assumption, deviation from a shared scaling relationship can therefore indicate an adaptive change in the functional relationship between two brain
structures (Montgomery, 2013). Correction for multiple testing was performed using a sequential Bonferroni procedure (Benjamini & Hochberg, 1995).

We used the same approach to analyse patterns of covariance between pairs of neuropils, to test whether volumetric differences occur in a concerted manner or result in non-allometric species-differences in the relationships between functionally-linked neuropils. This was supported with multiple regressions across larger groups of neuropils. Here, each neuropil of interest was regressed against functionally related neuropils in either the optic lobes or central brain. This analysis aimed to test whether the volumetric differences observed between species are independent of one another, whilst also controlling for total brain size.

All multivariate and allometric analyses were performed on i) wild individuals only; and ii) insectary-reared individuals only. All individuals from both groups were subsequently combined to test for the ability to identify species differences independently of group effects (wild/insectary) using a multivariate approach (PCA/DFA). We complimented these results with an ANOVA of the full dataset to test for independent species and group effects, and to test for group-by-species interactions.

Results

Divergence in brain composition

A Principal Component Analysis (PCA) revealed marked divergence in brain composition between the two species despite a lack of evidence for a difference in the overall volume of the central brain ($t_{30} = 0.688$, $p = 0.497$) or total neuropil volume ($t_{30} = 0.705$, $p = 0.487$; Figure S1). Using volumetric data for 13 neuropils and rCBR from 32 wild individuals (Supporting Information B Table S1), our PCA resulted in four major Principal Components (wPC), together explaining a total of 78% of total variance. Of these, wPC2 (18.574% Var; $F_1 = 33.840$, $p < 0.001$) and wPC4 (8.196% Var; $F_1 = 9.691$, $p = 0.004$) were significantly associated with species identity (ANOVA controlling for sex) (Figure 2A; Supplementary Results). This result is supported by a Discriminant Function Analysis (DFA), where the two species were separated along a single significant Discriminant Function (DF) (Wilks $\lambda = 0.165$, $\chi^2 = 39.664$, $p < 0.001$; Fig 2B) with 87% of individuals assigned to the correct species
group with a high degree of confidence ($p < 0.05$; Table S2A), a further 9.7% were correctly assigned but with lower confidence. Re-analysis with four groups (species + sex), or on males and females separately, also provide support for robust species differences (Online Supporting Information A Figure S2). Across these multivariate analyses, components of the visual pathway including the medulla, lobula and lobula plate, which are involved in processing of light, color and movement, and the antennal lobe, the primary olfactory structure in the insect brain, had consistently strong contributions to PCs or DFs that separate *H. himera* and *H. erato* (Table S2B).

To further explore how individual neuropils contribute to species differences in brain composition, we next examined the scaling relationship between each neuropil and an independent measure of overall brain size (the unsegmented volume of the central brain; rCBR). The majority of neuropils in the optic lobes display non-allometric shifts in scaling with rCBR between species (Table S4). After correcting for multiple tests using the false-discovery rate (Benjamini & Hochberg, 1995) (for 13 neuropils), both the medulla (FDR-$p < 0.001$) and lobula plate (FDR-$p = 0.026$) show significant grade-shifts between species (difference in $\alpha$), whilst the lobula shows a species difference in $\beta$ (FDR-$p = 0.026$) (Figure 2C-D). There is also some support for the accessory medulla displaying a grade-shift (nominal-$p = 0.044$). In all cases these differences result in an increase in the size of these structures in *H. erato*. In contrast, we identified two central brain neuropils, the antennal lobe (FDR-$p = 0.042$) and the posterior optic tubercule (FDR-$p < 0.001$), which show grade-shifts towards larger volumes in *H. himera* (Figure 2E-F).

We further explored species differences in the volume of the two major components of the antennal lobe; the glomeruli, which are innervated by axons from olfactory sensory neurons in the antennae (Hansson & Stensmyr, 2011), and the antennal lobe hub, which contain neuronal fibers from local neurons and projection neurons that connect glomeruli with each other and with other brain regions (Malnic *et al.*, 2000). Relative to central brain size, both the antennal lobe glomeruli (Wald $\chi^2 = 5.674$, $p = 0.017$) and hub (Wald $\chi^2 = 11.106$, $p < 0.001$) are expanded in wild *H. himera*, whilst maintaining a constant scaling relationship ($\beta$-shift LR $< 0.001$, $p = 0.991$; $\alpha$-shift Wald $\chi^2 = 0.940$, $p = 0.330$).

The results of our scaling analyses are largely consistent regardless of whether sexes are pooled or considered separately, or whether rCBR or an alternative measure of overall size (total neuropil minus the neuropil of interest) is used (see
Supplementary Results and Table S4 in Supporting Information B). Importantly, because neither rCBR ($t_{30} = 0.688, p = 0.497$) or total neuropil volume ($t_{30} = 0.705, p = 0.487$; Figure S1) vary between species, these differences represent changes in the volume of individual neuropil, not a concerted size change affecting all neuropils equally, or a shift in rCBR volume. These interspecific differences in scaling relationships also reflect substantial differences in volume. For example, for a given brain volume the medulla and lobula will be, on average, 12.3% and 18.2% larger in *H. erato* respectively, whilst in *H. himera* the antennal lobe will be 14.5% larger and the posterior optic tubercle 22.6% larger.

Covariance between brain components

Our analyses demonstrate that at least three of the six neuropils in the optic lobes are larger in *H. erato*. These neuropils process visual information and are both functionally interdependent and physically connected by projection neurons (Borst, 2009; Kinoshita et al., 2015). It is therefore a reasonable possibility that if one component expands, this would have knock-on effects on other neuropils. We analysed patterns of covariance between visual neuropils using linear multiple regressions (controlling for species and sex) to assess whether the change in scaling relationships for multiple individual optic lobe neuropils reflects independent differences in volume. This revealed that the six neuropils form a co-varying network (Figure 2G), partially reflecting patterns of functional inter-connectivity in other insects (Borst, 2009). After accounting for this covariance, the association with species only remains significant for the medulla (Table S5). This suggests changes in medulla size may be driving changes elsewhere in optic lobe, potentially contributing to species differences in other optic lobe components.

We further investigated this possibility by examining the pairwise scaling relationships between medulla, lobula, lobula plate and accessory medulla. Consistent with the conclusion that variation in the size of the medulla may drive changes in lobula plate size, these two neuropils show a major-axis shift between species along a conserved scaling relationship ($W^2 = 5.105, p = 0.024$). However, there is also evidence of species differences in scaling exponent between the lobula and both the medulla and lobula plate (Likelihood Ratio = 12.275, $p < 0.001$ and LR = 5.039, $p = 0.025$ respectively). The accessory medulla volume shows a grade-shift in scaling with the medulla, lobula and lobula plate, consistent with a lesser effect of species
identity on this neuropil (all p < 0.001; Table A5). These analyses suggest that the species differences in size of the medulla and lobula plate may constitute a concerted shift, maintaining but expanding their functional relationship, whilst altering their functional association with the lobula.

We identify one co-varying network amongst components of the central brain; between antennal lobe volume, the mushroom body lobes and the mushroom body calyx (Table S5B). This may reflect the well-established role of the mushroom bodies in olfactory learning (Heisenberg, 2003). We found no significant association between antennal lobe and posterior optic tubercule volume, or between either of these neuropils and medulla (Table S5C), suggesting that these reflect structurally independent shifts.

**Plasticity does not explain species differences**

The preceding analyses focus on wild caught individuals. However, *Heliconius* brains show significant amounts of environment-dependent and independent post-eclosion growth (Montgomery et al., 2015). To test whether environmentally induced developmental plasticity can explain the observed species differences we next analysed our dataset of an additional 10 individuals of each species reared in a common environment (see methods). A PCA of all neuropil volumes in insectary-reared individuals separated the variance in brain composition across 4 PCs (iPC), together explaining 73% variation in the dataset. Of these, iPC1 (35.068% Var, F₁ = 9.887, p = 0.006) and iPC2 (17.672% Var, F₁ = 17.672, p = 0.001) were significantly associated with species identity. Similar results were obtained when wild and insectary reared individuals were analyzed together (Figure 3A; see also Figure S5, Table S8).

We assessed whether the neuropils contributing to these iPCs were the same as those contributing to wPC2 and wPC4 in the wild caught samples by using a regression analysis of the loading coefficients for each neuropil. Loadings of neuropils on iPC1 from the insectary-reared analysis were significantly associated with loadings on both PCs associated with species from the wild analyses (wPC2: t₀ = 3.438, p = 0.007; wPC4: t₀ = 2.440, p = 0.037). Loadings on iPC2 were also significantly associated with loadings on wPC4 (t₀ = -3.223, p = 0.001) but not wPC2 (t₀ = -1.309, p = 0.223). Neither iPC1 or iPC2 show any association with wPC1 or wPC3 which do not vary consistently between species (all p > 0.100). A DFA also
provides strong support for species differences (Wilks $\lambda = 0.028$, $\chi^2 = 39.456$, $p < 0.001$) and assigns 100% of insectary-reared individuals to the correct species group with a high degree of confidence ($p < 0.001$; Table S6B). The DF coefficients again implicate the visual neuropils and posterior optic tubercule as contributors to this difference (Table S6). Together, these collective results strongly imply that the relative contribution of each neuropil to species differences in brain composition in the comparison between insectary-reared individuals is similar to that between wild individuals.

Further analyses of the scaling relationships between each neuropil and rCBR largely confirm this conclusion. We identify the same grade-shifts towards larger volumes in *H. erato* in medulla, lobula and lobula plate, and also in two further neuropils in the optic lobes; the lamina and ventral lobe of the lobula (all FDR-$p < 0.05$; Table S7A). However, grade-shifts against central brain volume are not found for the antennal lobe or posterior optic tubercule. Although these are recovered using total neuropil volume as an alternative variable, this may indicate some contribution of species differences in plasticity to the results uncovered in wild individuals (Table S7B). To explore this possibility we repeated the multivariate analyses combining wild and insectary reared individuals. In both a PCA and DFA we recovered a significant signal of species differences, as well as significant group (wild vs. insectary) effects (Figure 3D, Figure S5, Table S8). The association between PC and species are robust to controlling for group and sex for two axes of variance (PC2 15.23% Var, $F_1 = 70.670$, $p < 0.001$; PC3 13.336% Var, $F_1 = 26.384$, $p < 0.001$; Table S8). Analysis of variance in individual neuropils, controlling for sex, group and total brain size, also support the species differences reported above (Table S9). Consistent group-by-species interactions were found for three neuropils (lamina, ventral lobe of the lobula, and mushroom body lobes and peduncle; Table S9), none of which are implemented in species differences in wild populations.

**Discussion**

Both inherited, and environmentally induced changes in brain structure and function can play an important role during population divergence. However, few empirical studies have tested the contribution of these sources of variation during the early stages of speciation. Our analysis aimed to test whether heritable differences in brain
structure are observed between very closely related species, offering a novel case study of whether brain evolution play a role in ecological divergence. *H. erato* and *H. himera* are parapatric, incipient species adapted to contrasting environmental conditions. This divergence is reflected by heritable differences in the levels of investment in key brain structures that influence the perception and processing of sensory information in insects.

**Are species differences adaptive?**

We have demonstrated consistent species differences in the size of specific brain components in both wild caught individuals, and individuals reared in common garden conditions. Whilst this demonstrates these differences have a major heritable component, it does not demonstrate these differences are the result of selection rather than drift. Experimental assays linking specific behavioural tasks to component volumes will be required to fully explore the functional and adaptive nature of the changes in brain composition we observe. However, we argue that the differential volumetric investment we observe are likely to reflect adaptive evolution of brain structure and function. First, non-allometric grade-shifts in scaling relationships between brain components are unlikely products of neutral evolution. Allometric scaling in the brain is generally interpreted as being representative of stabilising selection to maintain the functional relationships between components of an integrated phenotype (Barton & Harvey, 2000; Montgomery, 2013). Instead, non-allometric shifts are often observed between groups of ecologically or behaviourally divergent individuals, reflecting a change in function or connectivity (e.g. Ott & Rogers, 2010; Farris & Schulmeister, 2011). Second, volumetric differences are caused by underlying changes in neuron size or number, both of which have significant energetic costs (Laughlin, 2001). Brain size and structure are therefore under strong selection for energy efficiency (Niven & Laughlin, 2008). Total brain size is similar across *H. himera* and *H. erato* which may imply a conserved overall energetic budget dedicated to brain development and function. It is possible the energetic costs of expanding one brain structure are balanced by reducing another, however, this will have functional costs. As such, changes in relative investment in different brain regions are unlikely under conserved selection regimes. Instead, differential investment in brain components reflects their relative importance in
sensory perception and processing, and the adaptive production of behaviour (e.g. Gronenberg & Hölldobler, 1999; Stöckl et al., 2016).

Finally, we note that our results mirror those found across more distantly related Lepidoptera with more radical differences in ecology. For example, the brains of nocturnal moths and diurnal Lepidoptera can be distinguished on the basis of differential expansion of the antennal lobe or medulla and lobula system which determine behavioural performance under different sensory tasks (Montgomery & Ott, 2015; Montgomery et al., 2015; Stöckl et al., 2016). Similarly, the Neotropical diurnal butterfly _Godyris zavaleta_, which is found in dark inner-forest has increased investment in the antennal lobe relative to _Heliconius_ or _Danaus_, which occupy habitats with greater light intensity (Montgomery & Ott, 2015; Montgomery et al., 2015). This further highlights the ecological relevance of the observed differences, and suggests similar selective pressures associated with divergent sensory environments may shape Lepidopteran brain composition across short and long evolutionary time-scales.

_Divergence in brain composition suggests a shift in the importance of different sensory modalities between incipient species_

We suggest that differences in habitat have led to a shift in investment in sensory modalities between _H. erato_ and _H. himera_. _H. erato_ invests in larger visual neuropils than _H. himera_, and in particular the medulla, lobula and lobula plate. These neuropils have specific roles in processing visual information. In other insects the medulla plays a role in the parallelization of photoreceptor signals (Borst, 2009) but also contains many processing elements with dual roles in color-vision and motion detection pathways (Morante & Desplan, 2004; Rister et al., 2007; Paulk et al., 2009). The lobula and lobula plate integrate visual information to extract abstract features such as shape and motion; for example the lobula plate is the primary site for motion computation and tracking in the optic lobe, whilst the lobula has been linked to escape and chasing behaviour (Hausen, 1984). Notably, the cellular architecture of the lobula plate is known to vary extensively across species in association with differences in flight behaviour (Buschbeck & Strausfeld, 1997). Given the difference in forest type inhabited by _H. himera_ and _H. erato_ the volumetric differences in these components may reflect contrasting demands of visual and/or spatial information related to the
density of vegetation and leaf size, and subsequent changes in light intensity and polarisation.

In contrast, *H. himera* has higher levels of relative investment in the primary olfactory neuropil, the antennal lobe. Antennal lobes are comprised of glomeruli that are innervated by axons from olfactory sensory neurons in the antennae, which express olfactory receptors (Hansson & Stensmyr, 2011). These glomeruli are arranged around the antennal lobe hub containing neuronal fibers from local neurons and projection neurons (Malnic *et al.*, 2000). The number of glomeruli is relatively constant across Lepidoptera (Montgomery & Ott, 2015), including *Heliconius* (Montgomery *et al.*, 2015). Across more distantly related butterflies variation in antennal lobe size is disproportionately due to variation in the volume of the antennal lobe hub (Montgomery *et al.*, 2015). This suggests the complexity and number of neuronal fibers from projection/local neurons in the antennal lobe hub, which may in turn be shaped by the complexity of the combinatorial interactions between glomeruli, dominates over changes in odor sensitivity, which is reflected by the volume of glomeruli (Montgomery *et al.*, 2015). The constant scaling relationship between the antennal lobe glomeruli and antennal lobe hub may suggest the foraging and/or reproductive behaviour of *H. himera* has a greater reliance on olfactory sensitivity, without changes in the complexity of olfactory repertoire utilised. The second striking expansion in *H. himera* is found in one of the smallest components of the central complex, the posterior optic tubercule. In other insects, this neuropil receives a variety of inputs, including visual information from the accessory medulla, and mechanosensory and chemosensory information from the antennal lobes and other body parts (Pfeiffer & Homberg, 2014). Although we did not find statistical support for covariation in antennal lobe and posterior optic tubercule volume, the expansion of the posterior optic tubercule could conceivably reflect an increased input from the antennal lobe.

In addition to these habitat differences, it is possible that *H. himera* and *H. erato* weigh sensory information differently during reproductive behaviour. Male choice in both species involves visual perception of color and color pattern, with both species mating assortatively (Merrill *et al.*, 2014), but is likely to also involve olfactory cues (Merrill *et al.*, 2015). In addition, species in the erato clade are ‘pupal-maters’, a reproductive trait where adult males locate female pupae to attempt to mate with freshly eclosed females (Beltrán *et al.*, 2007). Whilst it is possible the species
differences we identify relate to changes in mate finding and preference behaviour, the contrasting habitats occupied by *H. himera* and *H. erato* are likely to have shaped behaviours such as these, by altering the sensory background under which these mating cues are received. Disentangling these effects will require further study. However, we note *H. himera* does not show a deficit in visual preference compared to *H. erato* in male mate choice tests (Merrill et al., 2014) suggesting they are no less able to discriminate color pattern as a reproductive cue.

*Brain anatomy, plasticity and behavioural change*

Plasticity in neural development and behaviour has been implicated in facilitating local adaptation and catalysing speciation by promoting survival in novel habitats (West-Eberhard, 2003; Sol, 2009; Pfennig et al., 2010; Schwander & Leimar, 2011; Snell-Rood, 2013). Recent studies suggest plasticity in brain development contributes to local adaptation of brain morphology in ecological morphs within species (Eifert et al., 2011; Gonda et al., 2011). However, plasticity has significant functional and energetic costs (Snell-Rood, 2013) and the net benefits may consequently be transitory (Thibert-Plante & Hendry, 2011). The ‘plasticity-first’ hypothesis therefore remains controversial, partly due to a lack of examples in wild populations (Levis & Pfennig, 2016).

In a behavioural context, Snell-Rood (2013) distinguished two forms of plasticity; activational, which occurs through the differential activation of common neuronal networks in different environments, and developmental plasticity, which occurs through the differential development of neuronal networks in response to alternative conditions (Snell-Rood, 2013). Our analysis specifically considers the possibility of developmental plasticity, as activational plasticity will not result in volumetric differences. We have demonstrated that the incipient species *H. himera* differs significantly in brain composition compared to its ancestral-proxy lineage, *H. erato*, despite ongoing hybridisation across the contact zone and gene flow between the two populations. The neuroanatomical differences we observe cannot be explained solely by developmental plasticity, suggesting the action of selection to refine brain structure to meet the particular demands of the high altitude, dry forest *H. himera* occupies. Although we do observe significant group effects, demonstrating plasticity in brain maturation, these do not explain the species differences we observe in brain composition. In addition the brain components with good evidence of species-by-
group interactions – potentially indicative of species differences in plasticity – are not those that show robust evidence of divergence. Whilst our common-garden experiment is unlikely to reflect the natural environment of the ancestral population, our results suggest that if plasticity in brain structure and function did facilitate the initial establishment of the ancestral *H. himera* population, selection has quickly acted to canalise any beneficial response. Given the close relatedness of these incipient species, our results suggest local adaptation dependent on a plastic response may be too costly, or functionally insufficient, to offer stable route to persistence over even modest evolutionary time periods. Indeed, a transient role for plasticity is predicted by some models of ecological speciation (Thibert-Plante & Hendry, 2011).

Levis and Pfennig (Levis & Pfennig, 2016) recently suggested a series of criteria to formally test the ‘plasticity-first’ hypothesis, these include demonstrating environmentally-induced plasticity in a proxy ancestral population and adaptive refinement of phenotypic traits. We suggest ecologically divergent taxon pairs of *Heliconius* may provide a useful case study to address these criteria. First, taxon-pairs exist at multiple levels of evolutionary divergence (Mallet et al., 2007; Merrill et al., 2011), permitting the relative contribution of plasticity to taxon-differences to be quantified across the ‘speciation continuum’. Second, although practical limitations have prevented such an analysis here, it should be possible to expose ancestral proxy populations, such as *H. erato*, to the environmental conditions of a derived population, such as *H. himera*. This will provide an opportunity to assess the direction and magnitude of environmentally induced response to the novel habitat type.

Finally, whether species differences in behaviour require changes in the neuronal circuitry, or can be facilitated by altered activation patterns in conserved circuits is a key question in evolutionary neuroethology. Previous work has demonstrated that behavioural differences between closely related species can be produced by both altered neuromodulation and by changes in the strength of a synaptic response within a neuronal circuit with a conserved cellular architecture (reviewed in Katz & Harris-Warrick, 1999; Katz, 2007). The volumetric differences we observe must be produced by changes at the cellular level, either through changes in cell production or changes in the branching pattern and connectedness of synapses within the measured neuropils. Although our analyses do not reveal the nature of these changes, volumetric differences may be indicative of altered neuronal circuitry to produce behavioural and sensory adaptations to contrasting habitat types. This may
be through the addition or subtraction of a particular cell type(s) (e.g. Finger, 1997; Truman & Ball, 1998; Witten & Truman, 1998) and/or novel patterns of connectivity (e.g. Balaban, 1997; Ventura-Antunes et al., 2013). Whilst we do not rule out more subtle differences in neural activation we suggest our volumetric differences imply changes in the cellular architecture of neuronal networks also plays a significant role in ecological adaptation and behavioural differences between species.

**Conclusion**

Speciation across environmental gradients requires local adaptation to distinct ecological niches (Rice, 1987; Nosil, 2012). By focusing on a pair of incipient Heliconius species we have demonstrated that this exerts selective pressures that reshape brain composition, resulting in significant non-allometric shifts in a specific suite of brain components. Our results demonstrate that even at the early stages of speciation, with ongoing gene flow (Jiggins et al., 1997; Supple et al., 2015), heritable divergence plays a major role in shaping species differences in brain structure. We argue these non-allometric changes were likely driven by selection for adaptive divergence, rather than being the result of drift. Whilst this hypothesis requires further testing, our results are consistent with the hypothesis that brain and sensory systems are an important component of ecological adaptation.

**References**


Mallet, J. 1989. The genetics of warning colour in Peruvian hybrid zones of Heliconius erato and H.


Figure Legends:

**Figure 1:** Distribution and ecology of *H. erato cyrbia* and *H. himera*. A) Color patterns and key distinguishing features of the habitats of *H. e. cyrbia* (left) and *H. himera* (right). B) Approximate distribution of *H. e. cyrbia* (blue) and *H. himera* (red) in southern Ecuador showing the two sample localities, Balsas Canton and Vilcabamba, and the location of the hybrid zone south of Buenavista (Bv) and Chaguarpamba (Cp), but north of Zambi (Za). Geographic ranges are based on published data from Rosser et al., (2012). C) Variation in altitude across a transect illustrated by the grey dashed line in B. D) 3D volumetric models of the neuropil measured in *H. e. cyrbia* (left) and *H. himera* (right) viewed from the anterior (top row) and posterior (bottom row). VN: Visual Neuropils including those in the optic lobe and central brain, AL: Antennal Lobe, CC: Central Complex, MB: Mushroom Body.
Figure 2: Divergence in brain composition in wild *H. e. cyrbia* and *H. himera*. A) Biplot between wPC2 and wPC4, which are both significantly different between species. *H. erato* are in blue, *H. himera* are in red. Males are filled circles, females are unfilled. B) Separation of species by Discriminant Function Analysis. Asterisks denote group means. C-F) Scaling relationships for all individuals between the central brain (rCBR) and the medulla (ME) (C), lobula (LOB) (D), antennal lobes (AL) (E) and posterior optic tubercule (POTU) (F). G) Patterns of covariance between neuropils in the optic lobe including the ME, LOB, ventral lobe of the lobula (vLO), lobula plate (LOP), accessory medulla (AME) and lamina (LAM). Significant covariance is shown by solid black lines, with those neuropils with significantly different scaling relationships with rCBR between species shown above (interspecific allometric shifts), and those associated with species in the multiple regression shown below (LMM ~ species). * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 3: Consistent signal of divergence between wild and reared individuals.

A) Biplot of PC1 and PC2 from a PCA of insectary reared individuals. *H. erato* are in blue, *H. himera* in red. Both PC1 (35.068%; Var; $F_1 = 9.887$, $p = 0.006$) and PC2 (17.300%; Var; $F_1 = 17.672$, $p < 0.001$) show significant differences between species. Asterisks denote group means. B-C) Regressions between the loadings of the measured neuropil on iPCs explaining species differences in reared individuals (x-axis) and wPCs explaining species differences in wild individuals (y-axis; wPC2 = green, wPC4 = grey). D) Biplot of a combined PCA of wild (filled circles) and insectary reared (unfilled circles) individuals. Both PC2 (15.230%; Var; $F_1 = 70.670$, $p < 0.001$) and PC3 (13.336%; Var; $F_1 = 26.384$, $p < 0.001$) show significant differences between species. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 
Table 1: Non-allometric scaling shifts between species

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<tr>
<th>Location</th>
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<th>Neuripil</th>
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1 OL: Optic Lobes, CBR: Central Brain, VN: visual neuropil, AL: Antennal Lobe, MB: Mushroom Body, CC: Central Complex