Mushroom body expansion and the cognitive ecology of *Heliconius* butterflies

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This thesis is submitted for the degree of  
*Doctor of Philosophy*

Trinity College  
January 2022
Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

It does not exceed the prescribed word limit set by the Degree Committee for the School of Biology.

Fletcher James Young

January 2022
Summary

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*Heliconius* butterflies, a Neotropical genus of approximately 50 species, exhibit a marked expansion of an insect brain structure called the mushroom bodies (MBs), which are 3-4 times larger than in other Lepidoptera, including closely related Heliconiini genera. MBs are known to play a role in learning and memory, particularly in olfactory contexts, however the relative cognitive capabilities of *Heliconius* remain unknown. The central objective of my PhD is to investigate the behavioural consequences of, and selective pressures that drove, expansion of *Heliconius* MBs. I have explored these questions by collecting comparative behavioural and neuroanatomical data across *Heliconius* and closely related Heliconiini.

To explore patterns of MB evolution in the Heliconiini, I conducted phylogenetic comparative analyses across a neuroanatomical dataset of 41 species, including 30 *Heliconius* and representatives of all Heliconiini genera. Phylogenetic generalised linear mixed modelling shows that within the Heliconiini, increased MB size is associated with the *Heliconius* genus, even when controlling for the size of the central brain, the antennal lobe and the medulla. Moreover, variable rates analyses indicate that the branch leading to *Heliconius* experienced a significant increase in the rate of evolution of MB size. But what drove this expansion?

There are two main adaptive hypotheses to explain this MB expansion. One is that it facilitates an improved shape learning and recognition of *Passiflora* host plants. To test this, I conducted shape learning assays across six Heliconiini species. However, *Heliconius* did not, as group, out-perform the outgroup species. In addition, I conducted geometric morphometric analyses on *Passiflora* leaf shape to determine the morphospace of host plants Heliconiini species exploit. There was no correlation between host plant morphospace and MB size. Together these findings suggest MB expansion in *Heliconius* is not associated with an improved ability for the visual identification of host plants.
The second relates to *Heliconius’* unique foraging strategy. *Heliconius* are the only Lepidoptera known to actively feed on pollen, which they collect from a limited number of relatively rare plants. In visiting these plants, *Heliconius* establish “traplines” – routes through the forest that they follow with a high degree of spatial and temporal fidelity, and which seemingly relies on an advanced spatial memory ability. Through a series of behavioural experiments, I show that *Heliconius* can learn the location of a food resource in a T-maze, in addition to outperforming non-*Heliconius* Heliconiini in long-term memory and non-elemental learning tasks – cognitive abilities assumed to be crucial for traplining. There was no difference, however, between *Heliconius* and non-*Heliconius* in a reversal learning task. Nonetheless, these results are consistent with the elaboration of the *Heliconius* MB being driven by the cognitive demands of trapline foraging for pollen.

Finally, I investigate the possible neural correlates associated with long-term memory performance by comparing the mushroom bodies of *Heliconius erato* and *Dryas iulia* individuals involved in the long-term memory assay with control individuals reared in non-learning environments, in addition to a group of freshly-eclosed butterflies. Overall, the mushroom bodies of *Heliconius erato* exhibited significantly more age- and experience-related plasticity than *Dryas iulia*. Importantly, an increase in synapse count was associated directly with visual learning in *Heliconius erato*. At an individual level, within *Heliconius erato*, but not *Dryas iulia*, increases in synapse density and count were correlated with improved recall accuracy.
Acknowledgements

I am deeply indebted to Stephen Montgomery for his supervision and support over the last four years. As a supervisor, Stephen has extended to me incredible dedication and patience in guiding me through this journey. It is an honour to be his first PhD student.

I am also grateful for the assistance of other members of the Evolution of Brains and Behaviour Lab. I thank Antoine Couto for training me in immunocytochemistry techniques and confocal microscopy, without which Chapter 6 would not have been possible. Francesco Cicconardi assisted majorly with Chapter 2 by producing phylogenetic trees and helping me with code for data processing. Amaia Alcade assisted greatly with the data collection for Chapter 6 by researching methods for automated cell counting and helping with manual image segmentations.

The rearing of caterpillars is incredibly time consuming, and Chapters 3 to 6 would not have been possible without the invaluable support of field assistants in Gamboa and France. Monica Monllor, Lina Melo-Flórez, Anaïs Larue and Priscila Moura all made significant contributions to this work through their help in the field. Wyatt Toure and Jessica Foley, with whom I collaborated with on related projects in Gamboa, also helped to share the burden of butterfly rearing. I want to thank the rest of the STRI community in Gamboa for providing a scientifically stimulating and socially-rich environment in Gamboa, Panama. Particular thanks go to Owen McMillan for his support both in making my life as easy as possible at the STRI insectaries and his excellent cooking, and Oscar Cruz for maintaining the insectaries.

I thank Guillermo Navalón for his support not just as a friend and housemate in the final six months of this process, but also for illuminating discussion on phylogenetic comparative methods and prescient aesthetic criticism of figures. Dale Buckley, in lockdown in Melbourne at the time, helped me make it through the wintry months of January to April 2021, while I was working alone in the labs and confocal room of the Bristol Life Sciences Building. While in Cambridge, I was fortunate to make many friendships that I hope to maintain for the rest of my life. This work would not be possible without their support.

I thank Teja Potocnik for providing the inspiration to get across the finish line in the final few days before submission.
None of this work would have been possible without financial support from Trinity College, Cambridge. I will be forever grateful to the College for extending me this opportunity.

Most importantly, I thank my parents, Gail and Mark, and sister, Bronte, for their incredible support and unconditional love. They have fully supported me through all my endeavours, without judgement.
Publications and Collaborations

The introduction of this thesis was partly based on a review of pollen feeding in *Heliconius* I published in *Proceedings of the Royal Society B*:


A copy of this article can be found in the Appendix.

Chapters 2 through 6 all involved substantial collaboration with others.

For Chapter 2, butterfly samples were collected, stained, and scanned by my supervisor, Stephen Montgomery, over several years. Brains were then segmented by Stephen Montgomery, Daniele Atzeni and myself. The phylogenetic comparative analyses for this Chapter relied on a tree generated by Francesco Cicconardi using newly assembled Heliconiini genomes.

The experiment in Chapter 3 was conducted in France using CNRS facilities, where Julien Cote provided valuable support. The running of this experiment was assisted by Monica Monllor and Priscila Moura.

Behavioural experiments in Chapters 4 and 5 were conducted at the Smithsonian Tropical Research Institute in Gamboa, Panama, where Owen McMillan provided important support. Monica Monllor (Chapter 4) and Lina Melo (Chapter 5) contributed significantly to these experiments as field assistants. Chapter 4 also utilised an unpublished dataset on Heliconiini host plant use collated by Krzysztof Kozak.

For Chapter 6, Antoine Couto designed the staining protocol and synapse counting methods, and trained me in immunohistochemistry and use of the confocal microscope. Amaia Alcade assisted in image processing by segmenting Kenyon cell clusters and using automated methods to count Kenyon cells. Jessica Foley assisted by dissecting several *Heliconius erato* individuals.
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1 Introduction

1.1 Brain size and cognition: a complicated relationship

Brain size varies remarkably throughout the animal kingdom, prompting long-standing assumptions about the relationships between brain size and cognitive ability [1,2]. An increase in relative brain size has been assumed to have a corresponding increase in cognitive function, owing to increased neural processing power [1,3,4]. Several studies have, indeed, found correlations between brain size and measures of “intelligence” [5–10]. However, the use of brain size in such comparative studies, and its merits as a representation of intelligence has also been criticised [4,11–13]. Such comparative studies may be undermined by several factors [4]. For example, brain size does not scale linearly or uniformly with body size across species [14–16]. Rather, allometric relationships between the brain and body are specific to particular groups [13,17–19], potentially undermining comparative studies across disparate groups. Furthermore, brains are composed of different regions dedicated to different functions, such as learning and memory, sensory processing, and motor control. Measures of whole brain size could fail to detect size variation in one of these areas as brain regions do not scale uniformly with total brain size [20,21]. Importantly, brain size does not uniformly scale with neuron number across groups due to significant differences in neuron density [22,23]. Additionally, although a single-factor understanding of general intelligence, mainly in the context of humans and other primates, has been advocated [24–28], its applicability to non-primates, particularly non-mammals, has been questioned [4,29,30]. One recent meta-analysis found weak support for general intelligence in animals, with low average correlation among cognitive abilities [30]. The authors conclude that reducing intelligence to a single dimension can mask important variability in specific cognitive abilities, possibly leading to misleading findings [30].

These issues are further complicated by the fact that brain structure itself evolves, a process which is subject to a complex interaction of selective pressures and functional and developmental constraints (discussed below). The coevolution of cognitive ability and brain size is influenced by interactions with life history and other ecological traits [31–35], while
developmental and functional constraints may limit the independent evolution of individual brain regions [36–38]. Importantly, brain evolution brain evolution should be viewed as being shaped by selection for specific, ecologically-relevant behaviours [39]. When comparing cognitive performance across species, poor performance in a given task should not necessarily be taken as evidence of overall cognitive ability [4,40–42]. These interdependencies between different brain structures, behaviour, life history and ecology can therefore complicate relationships between brain size and cognition, potentially obscuring the selective pressures shaping the brain. Therefore, there are good reasons to question the assumption that a generally bigger brain necessarily translates into an increase in cognitive ability [43]. Considering brain size alone can miss crucial detail about its underlying structure and neuronal connectivity [4,11,12].

Many of the issues flagged above can be avoided by conducting comparative studies focusing on linking specific brain regions to certain behaviours or cognitive abilities [44]. Studies comparing individuals within a species, or from closely related species, can more precisely identify the neural mechanisms underlying certain behaviours, and as a result, inform on the putative selective pressures acting upon a particular brain region. Finally, because brains evolve in response specific ecological demands, it is important to assess cognition is a way that is ecologically relevant, and which aims to test specific adaptive hypotheses about the brain [45].

1.2 The drivers and constraints shaping brain evolution

The nervous system plays an essential role in mediating an animal’s interactions with its environment, both receiving and processing sensory input, and controlling the motor response to those stimuli. Brain evolution is, thus, shaped by the cognitive demands imposed by an animal’s ecology, including foraging behaviours [46–48], mate detection and selection [49], and predator avoidance [50]. However, there are substantial energetic costs associated with neural tissue [51–54], which can constrain how brain evolution is shaped by these selective pressures. Neural elaboration will, therefore, only occur when the benefits of increased neural investment outweigh its costs. Importantly, the relative rewards of neural investment can vary significantly with life history traits, and neural elaboration is positively correlated with longer lifespans [55–57]. According to the “delayed benefits” hypothesis, investment in learning,
long-term memory and their neural correlates is likely to be less rewarding for shorter-lived species [58]. Investment in a larger brain imposes a substantial metabolic cost, while the benefits of learning and memory accumulate over time, and it may only be in longer-lived species that this investment “pays off”. Over a longer lifespan, animals will also likely be exposed to more environmental variability, where learning and behavioural flexibility could be expected to be even more rewarding [58]. Evidence for such a pattern has been found in rodents, where longer lifespans appear to have enabled the evolution of larger brains [55].

Additionally, the independent evolution of component parts of the brain may be limited by developmental and functional constraints arising from its structural framework [36–38]. The extent to which different regions of the brain can evolve independently is subject to considerable debate [36]. The “mosaic” model of brain evolution argues that separate regions of the brain can evolve independently in response to selective pressures, being primarily constrained by the functional connectivity of brain regions [17,20,59,60]. Conversely, the “concerted” model of brain evolution argues that developmental mechanisms significantly limit the extent to which separate brain regions can evolve independently, with changes in the duration of neurogenesis shifting the size of all brain regions in concert [61,62]. These models do not preclude each other, but their relative importance in determining variation in brain structure continues to be debated [63]. Although such debates are generally discussed with reference to vertebrate evolution, developmental constraints [64,65] and examples of mosaic brain evolution have also been identified in insects such as the marked expansion of the mushroom bodies within Hymenoptera [21] and a reduction of the olfactory lobes within the nocturnal wasp Aopica pallens [66].

One way of teasing apart these various factors influencing brain evolution, and understanding the conditions under which neural elaboration is favoured by selection, is by conducting comparative neuroanatomical and behavioural studies across closely related species [6,67,68]. Of particular interest is the evolution of integrative brain centres, for example the hippocampus of vertebrates [69], the cerebral cortex of mammals [70], and the mushroom bodies of insects [71]. These regions receive inputs from sensory processing centres and play an important role in cognitive functions such as learning and memory. The hippocampus [17,72], the neocortex of primates [20] and the insect mushroom bodies [66] all provide examples of mosaic evolution. The expansion of the hippocampus in food-storing birds [17,72–76], where it has been linked to improved spatial memory [77–82], stands as a particularly well-studied example of a mosaic shift in the brain with corresponding cognitive effects.
1.3 The insect mushroom bodies

The mushroom bodies are paired, central structures in the insect brain that receive visual and/or olfactory information, and play an important role in learning and memory [83]. Mushroom bodies are formed by intrinsic neurons called Kenyon cells [84], and consist of three main regions: the calyx, the lobes, and the peduncle connecting them. The calyces are made of dendrites from the Kenyon cells, which then provide an axon to the peduncle, which extends to the lobes [85]. Depending on the species, the calyces variously receive olfactory and/or visual inputs, from the antennal and optic lobes respectively [86]. The calyx is comprised of synaptic complexes, called microglomeruli, formed by the presynaptic bouton of a projection neuron from the antennal or optic lobe and postsynaptic endings of Kenyon cell dendrites [87]. Microglomeruli density and number can vary with both age and experience, is likely associated with memory formation [88–92].

Among insects, the mushroom bodies vary substantially within this basic plan. For example, honeybee mushroom bodies feature a pair of prominent, well-defined calyces, while those in *Drosophila* are relatively small. Strikingly, in certain, mostly aquatic, species, the calyx is entirely lost [93]. This variation in structure likely reflects variation in function, as different insect groups face different selective pressures, rely on different sensory modalities, and vary dramatically in life history, longevity and therefore the relative benefits of innate and learned behaviours [86]. One important variation in mushroom body structure is the relative importance of visual and olfactory input to the calyx [86]. The mushroom bodies are most widely studied as olfactory learning and memory centres, primarily in *Drosophila* [88,89,94–96]. However, in other insect groups, the mushroom bodies variously play a role in visual learning and memory [90], spatial navigation [97–99], reversal learning [100,101], and non-elemental associative learning [102]. Interestingly, expansion of the mushroom bodies has independently occurred in several different insect lineages occupying a range of ecological niches, including Hymenoptera [21], cockroaches [103,104], and beetles [105,106]. This makes the mushroom bodies an interesting case study in understanding the processes and mechanisms that lead to mosaic patterns of brain evolution.
Chapter 1 – Introduction

1.4 Insect cognition, the mushroom bodies, and *Heliconius* butterflies

In a field traditionally dominated by studies on vertebrates, insects have emerged as valuable models for studying the evolution of cognition [44,107–109]. Insects are capable of surprisingly sophisticated cognitive feats, including facial recognition [110,111], counting ability [112,113], tool use [114,115], social learning [116,117], and abstract concept learning [118,119]. In addition, insects can also exhibit attention-like processes [120] and emotion-like states manifesting as pessimistic biases [121], and use of internal models in controlling movement [122]. These studies clearly demonstrate that complex behaviours can be supported by the miniaturised brains of insects. The navigational abilities of insects, in particular, have been the subject of considerable research interest [123,124]. For example, orchid bees can remember locations several kilometres apart [125], bumblebees can remember efficient routes linking multiple sites [126], and ants exhibit similarly impressive navigational abilities [127], even navigating across featureless terrain using path integration [128].

Often, these more sophisticated cognitive behaviours of insects are speculated to be supported by the mushroom bodies [21,83,111,112,119,129], however, in many cases these causal links remain untested. One of the difficulties arises from many of the behavioural experiments being carried out only in species with enlarged mushroom bodies, particularly a limited number of Hymenoptera. In Hymenoptera, mushroom body expansion is estimated to have occurred approximately 150 million years ago [21], making it difficult to conduct meaningful comparative behavioural experiments with related taxa that test specific functional and adaptive hypotheses. Ablation studies that impair the mushroom bodies have provided important insight into mushroom body function [97–99], but comparative behavioural experiments studying closely related species varying in mushroom body size are crucial to properly understanding the mushroom body in the context of cognitive evolution.

The Neotropical butterfly genus *Heliconius* (Nymphalidae: Heliconiinae) provides a novel and potentially powerful system for exploring this topic in a comparative framework. A further example of mushroom body expansion has also been identified in *Heliconius* butterflies. Data from three *Heliconius* species suggest that their mushroom bodies are 3-4 times larger than the closely-related Heliconiini species, *Dryas iulia* and *Agraulis vanillae* [129,130]. *Heliconius* diverged from their sister genus *Eueides* relatively recently, approximately 18 million years ago, and from other Heliconiini around 26 million years ago [131]. This provides the opportunity to investigate the expansion of a higher-order brain region over a relatively
small phylogenetic scale using ecologically similar species. Crucially, there are plausible hypotheses that could explain why mushroom bodies are expanded in *Heliconius*, and existing data on their ecology and phylogenetic relationships provides the framework to test these hypotheses. Notably, the apparent expansion of the mushroom bodies in *Heliconius* seemingly co-occurs with the emergence of a foraging strategy unique amongst Lepidoptera – pollen feeding [132]. This behaviour is associated with a suite of seemingly co-adapted traits, including an apparent increase in behavioural sophistication [133,134].

1.5 Pollen feeding in *Heliconius* butterflies: Brains, behaviour, and the evolution of adaptive suites

Major evolutionary transitions, including neural elaborations, can be triggered by behavioural novelty [135] and are often associated with “adaptive suites” that incorporate multiple co-adapted traits [136]. These transitions involve complex interactions, including mutual dependency between traits [136]. In these circumstances, disentangling the causal relationships between behavioural, neuroanatomical and morphological traits involved in major transitions is challenging.

The Neotropical butterfly genus *Heliconius* presents an excellent system for investigating how adaptive suites evolve. Uniquely among butterflies, *Heliconius* supplement their nectar diet by actively collecting and feeding on pollen [132]. *Heliconius* gather pollen by probing flowers and collecting it as a lumped mass on the proboscis (Figure 1.1(a)). The pollen load is mixed with saliva and externally digested to release amino acids that are then drawn up the proboscis [132]. Pollen is collected primarily from a limited number of plant species [137], and *Heliconius* from at least the *melpomene* clade (Figure 1.1(g)) show a particular preference for certain pollen-rich cucurbitaceous vines [137–139], with which they are hypothesised to have co-evolved [133,134].

This dietary innovation provides adults with a consistent supply of amino acids, permitting a prolonged reproductive lifespan [140], and co-occurs with behavioural, neuroanatomical, life history, physiological, and morphological changes that are absent in closely-related, non-pollen-feeding Heliconiini [141] (Figure 1.2 & 1.3). Among these putative adaptations, apparent specialisations in foraging behaviour have received particular attention.
*Heliconius* establish “traplines”, foraging routes along which specific plants are regularly visited, suggesting a sophisticated capacity for spatial navigation, likely using learned visual landmarks [133,142,143].

Pollen feeding evolved relatively recently, with *Heliconius* and its sister genus *Eueides* diverging *circa* 18 million years ago. However, the suite of traits associated with pollen feeding is widely assumed to have evolved only once [144] (Figure 1.1(d)-(g)), presenting difficulties in separating evolutionary cause and effect. As such, many hypothesised links between different aspects of *Heliconius* biology are poorly tested. Moreover, no physiological, morphological, or molecular traits have been specifically linked to the origin of pollen feeding in this genus. Nevertheless, as a major system for studying the genomic bases of adaptation [141], considerable genomic resources have been developed for *Heliconius* [145]. Together with recently developed genetic techniques and comparative methods [146–148], this provides a clear route towards understanding the evolution of pollen feeding and associated innovations in *Heliconius* butterflies.

Additionally, investigating the evolution of pollen feeding in *Heliconius* can contribute towards central questions in evolutionary biology, including:

i. how dietary shifts can alter energetic constraints, re-shaping life history and reproductive trade-offs;

ii. how evolutionary innovations co-opt ancestral molecular and morphological traits;

iii. and most importantly for the present work, the factors shaping neural elaboration, and its behavioural consequences.

In the following sections, I discuss the significant adaptive benefits of pollen feeding in terms of its life history effects and dependence on novel, sophisticated behaviours, which are expected to be supported by neural elaboration.
Figure 1.1 Evolution of pollen feeding. (a) A captive *H. hecale* with pollen load (white) affixed to its proboscis. (b) Elongated sensory bristles on the proboscis of *H. melpomene*. (c) A 3D reconstruction of a *H. hecale* brain showing the mushroom bodies (red) and rest of the brain (grey) from the posterior (top) and anterior (bottom) view. Scale bars: 500 μm in (b) and (c). (d)-(g) How phylogenetic hypotheses for Heliconiini have changed through time, and how that affects predicted gains (red circle/square) and loss (black circle) of pollen feeding. Pollen feeding lineages are shown in red, dotted lines indicate branches with higher uncertainty. Note the changing position of the Neruda clade, a group of four non-pollen feeding species. (d) Adapted from [149] and based on morphological data. (e)-(g) Adapted from [131,144,150] and based on molecular data.

1.6 Pollen proteins: an energetic payload

Populations are often under energetic constraint, resulting in investment trade-offs between competing tissues, traits, or strategies [151]. Shifts in diet quality have dramatic effects on evolutionary trajectories by increasing individual energy budgets. For example, dietary innovations have been linked to larger body sizes in carnivores [152], extended life spans in parrots [153], and increased reproductive investment in butterflies [154]. Applied to the evolution of the brain, a metabolically costly organ, this concept has been termed the “Expensive Brain Hypothesis” [33,51], and, indeed, changes in diet have been linked to brain expansion in primates [47,155–157]. However, such comparative life history studies often
compare phylogenetically and ecologically diverse species, leading to difficulties in
determining the precise role of dietary shifts in macroevolutionary dynamics (see [158]).

Like most holometabolous insects, Lepidoptera experience a profound shift in nutrient
intake between the larval and adult stages. Lepidopteran larvae are generally herbivorous,
whereas adults of most species feed predominantly on nectar (likely the ancestral condition
within butterflies [159]), which is carbohydrate-rich, but protein-poor [160–163].
Consequently, reproductive output is largely constrained by protein acquired during the larval
stage [154,160]. While some Lepidoptera also exploit alternative nutrient sources including
mud, fruit, dung, and carrion [161,164–168], pollen feeding in Heliconius is perhaps the
clearest example of a change in adult diet being linked to major shifts in life history. Pollen
feeding provides Heliconius with a consistent source of essential amino acids during the adult
stage [132]. Compared to other Heliconiini, Heliconius exhibit a pronounced delay in female
reproductive senescence [140]. Heliconius females generally collect more pollen than males
[138,169], incorporating pollen-derived amino acids into eggs [170], and suffer a marked
decline in fecundity when deprived of pollen [140]. This reflects a potential physiological
convergence with honeybees, where colonies cease brood rearing when denied access to pollen
[171].

Heliconius butterflies also have dramatically extended lifespans, living for up to six
months in the wild, without diapause [132,142], compared to a maximum of 4-6 weeks in
Dryas iulia, a non-pollen-feeding Heliconiini [140]. However, the causal relationship between
pollen feeding and longevity is not well quantified. One study reports that pollen-deprived H.
charithonia are shorter-lived [140], but this difference was not tested statistically. In adult
honeybees pollen feeding is associated with increased longevity [172], providing reason to
expect a similar effect in Heliconius. Interestingly, lifespans comparable to Heliconius have
been recorded in some fruit-feeding butterflies [173], raising the possibility that fruit-derived
amino acids may similarly facilitate an extended lifespan.

The energetic payload provided by pollen feeding has therefore clearly had a major
impact on reproductive output in Heliconius, and likely also lifespan. Such life history shifts
have the potential to favour selection for increased investment in neural tissue [55–57].
Similarly, the apparent lifting of metabolic constraints granted by access to pollen may also
have allowed for greater investment in neural tissue [33,47]. However, a proper understanding
of brain evolution in *Heliconius* requires consideration of the emergence of pollen feeding as a novel trait and its associated suite of putatively adaptive traits (Figure 1.2).

### 1.7 Origins of pollen feeding in *Heliconius*

The processes and underlying conditions that give rise to evolutionary novelties are incompletely understood [174]. Although evolutionary novelties can arise from the emergence of new ecological opportunities [175], this is not always the case [176]. Importantly, evolutionary trajectories appear constrained by pre-existing variation [177,178], suggesting contingency plays a substantial role in the emergence of novel traits. For example, complex behaviours can be achieved through the integration of simpler, pre-existing behavioural modules [179]. However, the relative importance of behaviour, morphology and physiology as the ultimate drivers of novelty is debated [174]. Understanding fitness benefits during intermediate stages, and the timing of trait acquisition, is therefore key to disentangling the origins of an evolutionary innovation.

**i) Reconstructing evolutionary shifts in pollen feeding**

Except for the four species of the ‘Neruda’ clade (Figure 1.1(e)-(g)), all *Heliconius* species feed on pollen and appear to possess the complete suite of associated traits, presenting a challenge to reconstructing the origin of pollen feeding. As the only non-pollen-feeding *Heliconius*, the four Neruda species may offer the possibility of decoupling pollen feeding and its associated adaptations, including the apparent expansion of the mushroom bodies, helping to resolve the timing of these shifts, and the relationships between traits. Regrettably, such analyses are currently limited by the scarcity of data on Neruda biology and lingering uncertainty regarding their phylogenetic position. Long considered a separate genus [149] (Figure 1.1(d)), recent molecular phylogenies have positioned Neruda within *Heliconius* [131,144,150] (Figure 1.1(e)-(g)). Hence, whether pollen feeding evolved once in *Heliconius* and was secondarily lost in the Neruda clade, or evolved multiple times within *Heliconius*, with Neruda retaining the ancestral state, is unclear, with these two scenarios being equally parsimonious (Figure 1.1(g)) [131]. In addition, given evidence of widespread introgression throughout the evolution of the genus [145], which could mislead the species tree, it also remains possible that the Neruda are, after all, a sister clade to *Heliconius*, as suggested by morphological data (Figure 1.1(d)). Nevertheless, the absence of pollen feeding in other
Heliconiini suggests that a single gain within *Heliconius* is likely, with or without a loss in the Neruda. However, discordance between any single Heliconiini species tree and underlying gene trees may present persistent difficulties in resolving this question [180].

More broadly, the scarcity of pollen feeding across the ~180,000 described species of Lepidoptera marks *Heliconius* as particularly peculiar. Although wild-caught butterflies from several genera have been reported as having pollen affixed to their proboscis [181], active pollen feeding is unknown in other Lepidoptera, with only a few exceptions, all of which are separated from *Heliconius* by large phylogenetic distances. Two families of basal moths, Heterobathmiidae and Micropterigidae, feed on pollen as adults [182]. These groups, however, lack a proboscis and use plesiomorphic mandibles to collect and crush pollen [182]. Two species of Gelechiidae moths are the only other proboscis-bearing Lepidoptera reported to feed on pollen, purportedly by dissolving the pollen wall with an unidentified salivary agent [183]. However, the ecology and life history of these species, and the prevalence of pollen feeding across Gelechiidae, are poorly understood, making it difficult to assess the feasibility of comparative analyses within this group.

**ii) Using old traits for new purposes**

Given the benefits of pollen feeding discussed above, it remains puzzling that pollen feeding is so rare. Central to answering why, is identifying the adaptations necessary for the transition to pollen feeding, including the relative importance of behavioural innovations and the potential role of mushroom body expansion. The apparent lack of associated major morphological adaptations with the emergence of pollen feeding in *Heliconius* is suggestive of behavioural innovation and associated neural elaboration playing a key role instead. Comparative studies across Heliconiini suggest that pollen collection does not involve any novel morphological structures [184]. However, *Heliconius* do have elongated proboscises compared to non-pollen-feeding Heliconiini, with longer and more numerous bristles at the proximal- and mid-regions (Figure 1.1(b)), which may assist in affixing pollen grains [184]. In addition, the intrinsic muscles involved in coiling movements are more numerous and extend further into the proboscis [185]. Pollen collection also involves the same sequence of probing movements as nectarivorous butterflies [161,186], although *Heliconius* do probe with higher frequency and handle individual flowers for longer, with handling time increasing in the presence of pollen [161,186]. Pollen processing may be derived from proboscis grooming
behaviours, which similarly involves the release of saliva and the repetitive coiling of the proboscis [187].

Saliva appears to play a key role in pollen feeding by helping to bind pollen to the proboscis and facilitating external digestion. Indeed, the salivary glands of Heliconius are larger than in other Nymphalids [188], and Heliconius release greater quantities of saliva during feeding [186]. Although the saliva of Heliconius contains proteases [189,190], it is unknown how it differs from that of other Heliconiini. However, proteolytic activity of the saliva does increase when the proboscis is stimulated with pollen, and is generally higher in females [189]. Two of the proteases identified in H. melpomene saliva also show close homology with the serine protease cocoonase [190,191], which is secreted from the proboscis of silkworms to weaken the cocoon during eclosion [192]. Like all butterflies, Heliconius lack cocoons, and it has been suggested that cocoonase homologs may have been co-opted for use in digesting pollen proteins [190,191], potentially weakening the pollen wall. Cocoonase-coding genes underwent several duplications along the lineage leading to Heliconius and their sister genus, Eueides, with an additional duplication specific to Heliconius and a further duplication in H. melpomene [191].

However, a functional role for cocoonase in pollen feeding has not yet been directly demonstrated. Importantly, it is unclear how proteolysis would break down sporopollenin, the primary component of pollen exines, as it is not composed of proteins [193]. Pollen grains are, in fact, visibly damaged after processing by Heliconius [194]. However, it is unknown if this is achieved solely by mechanical digestion, or through biochemicals capable of breaking down sporopollenin, as is claimed for Gelechiidae moths [183]. The role of specific salivary proteins in pollen digestion therefore requires further comparative studies that include a broader representation of the non-pollen-feeding Heliconiini.

Therefore, pollen feeding appears facilitated, in part, by the evolutionary co-option and modification of pre-existing anatomical, behavioural, and physiological traits for new purposes. However, the novel, sophisticated foraging strategy employed by Heliconius, and accompanying neural elaboration, seems crucial to understanding the emergence of pollen feeding as a successful foraging strategy.
1.8 Increases in behavioural sophistication and neural elaboration

Dietary innovations not only involve adaptations in the processing and utilisation of a resource but often co-occur with changes in foraging behaviour, as determined by the quality, and spatial and temporal distribution of food sources [195,196]. These parameters impose demands on perception, learning, and memory, and can favour investment in associated brain structures [46]. In Heliconius, the interactions between butterflies and their pollen sources may have led to notable refinements in both the brain and behaviour [129,130,133,143]. Heliconius therefore offer a highly tractable system for investigating how behavioural innovations can alter a species’ cognitive ecology.

i) Exploitation of a novel resource: plant-animal interactions and foraging strategies

Many species of Heliconius collect pollen predominantly from cucurbitaceous vines, particularly the relatively rare, but pollen rich, Psiguria and Gurania, which show evidence of co-evolution with Heliconius [133,134,137–139]. Heliconius are the primary pollinators of Psiguria, visiting more plants, and depositing more pollen, over greater distances, than even hummingbirds [197]. Indeed, several species of Gurania and Psiguria appear to have evolved lower nectar production and smaller flowers to promote visitation from Heliconius over hummingbirds, and older plants may even switch from producing male to female flowers once Heliconius visitation is established [198].

Psiguria flowers contain large amounts of pollen, and inflorescences generally produce a new flower every one to three days [133]. Individual plants, and even individual inflorescences, can flower continuously for up to a year [133], in contrast to the seasonal pollen production common for neotropical angiosperms [200]. A single Psiguria plant is therefore potentially a reliable pollen resource for the entire lifespan of an individual. Heliconius utilise
this dependable, but scarce, resource by establishing “traplines”, foraging routes along which specific plants are visited with a high degree of spatial and temporal regularity [133,142,143]. This suggests *Heliconius* possess a capacity for navigation using learned visual landmarks, similar to behaviours observed in certain bees [201,202]. *Heliconius* traplines centre on a limited home range of 100 m$^2$ to 1 km$^2$, within which individuals return to the same roosting locations at night, located using visual cues [143,197,203]. Although other butterfly species, including the heliconiine *Agraulis vanillae*, are reported to temporarily establish home ranges [204], *Heliconius* seem peculiar in maintaining long-term, stable home ranges with high roost-site fidelity [143,197,205]. Experimentally translocated *Heliconius* quickly orientate towards, and return to, their site of origin after release [205]. Site fidelity is presumably a pre-requisite for traplining, which, together with the central role of pollen resources in *Heliconius* traplines, suggests that these behaviours are linked to the acquisition of pollen feeding. However, the lack of data on whether non-pollen feeding Heliconiini use spatial information during foraging means this hypothesis is yet to be formally tested.

Despite the role of *Psiguria* and *Gurania* in *Heliconius* foraging behaviour, there is considerable variation in the exploitation of pollen sources between *Heliconius* species. Although these differences are partly explained by habitat preference, there appears to be a division between the two main *Heliconius* clades [137]. Species of the *melpomene* clade tend to forage more intensively on *Psiguria*, while *erato* clade species predominantly exploit non-cucurbitaceous plants, such as *Lantana* [137–139] (Figure 1.1(g)). Notably, *H. erato* do trapline on specific patches of *Lantana* [143]. Hence, the role of cucurbitaceous pollen sources in the transition to pollen feeding is unclear. One possibility is that pollen feeding arose in the context of coevolution with certain cucurbitaceous vines, with members of the *erato* clade subsequently pushed towards other pollen resources due to competitive exclusion. Alternatively, pollen feeding may have originated as a more opportunistic, generalist strategy, retained in the *erato* clade, with specialisation on Cucurbitaceae secondarily emerging in the *melpomene* group.

Many aspects of the foraging behaviours of *Heliconius* remain relatively poorly understood. For example, we do not know how traplines are established, the number of food sources utilised, the extent to which traplines change over time and how host plants may be incorporated into routes, as is purported by Gilbert (1975). Crucially, very little is known about the foraging behaviours of non-pollen-feeding Heliconiini, and whether they also engage in traplining behaviour.
ii) Neural basis of a cognitive adaptation: mushroom body expansion in *Heliconius*

Behavioural innovations are generally associated with changes in the structure and function of the brain [4]. For example, foraging innovations are linked to brain expansion in several vertebrate groups, including primates [47,155], birds [48,73], and bats [206]. Trapline foraging in *Heliconius* represents a degree of behavioural sophistication rarely reported among the Lepidoptera, and is suggestive of enhancements in visually-oriented spatial memory and long-term memory retention [133]. The apparent cognitive demands of traplining are predicted to be associated with elaborations in the *Heliconius* nervous system [129,130,207].

Indeed, data from three *Heliconius* species show dramatically enlarged mushroom bodies (Figure 1.1(c)), which are three to four times larger than is typical of Lepidoptera, including two closely related Heliconiini, *Dryas iulia* and *Agraulis vanillae* [129,130]. In *Heliconius*, the cognitive demands of trapline foraging, particularly an enhanced spatial memory, are hypothesised to have driven mushroom body expansion [129,130]. Supporting this hypothesis, two recent ablation studies in ants have directly implicated the mushroom bodies in visually-oriented spatial navigation [98,99], complementing an older study in cockroaches [97]. Indirect evidence also comes from comparative data on Hymenoptera, showing that expansion of the mushroom bodies coincided with the evolution of parasitoidism [21], which relies on spatial memory for host location [208]. This is complemented by plasticity experiments in a desert ant that show visually-guided foraging experience affects mushroom body maturation [209]. Nevertheless, these data are relatively impoverished compared to our understanding of the role of the central complex, another sensory-motor integration structure in the central brain, in insect spatial learning and orientation [210,211]. In fact, visual spatial learning in *Drosophila* does not require the mushroom bodies at all [212].

Increases in *Heliconius* mushroom body size have also been speculatively linked to host plant use [213]. *Heliconius* lay eggs exclusively on *Passiflora* plants, with varying degrees of specialisation. *Passiflora* display a remarkable diversity of leaf shape, and host plant use in *Heliconius* appears to be, in part, based on leaf shape recognition and learning through associative conditioning [214]. Mushroom body expansion may, therefore, support a greater array of search images and enhanced shape-learning abilities, facilitating improved visual identification of host plants [213]. Indeed, there are indications that, for some butterflies, mushroom body plasticity is shaped by experience with host plants [215,216]. However, the current lack of data on variation in mushroom body size within *Heliconius* and across
Heliconiini prohibits a formal testing of these hypotheses. Likewise, a better understanding of the foraging behaviours of non-pollen-feeding Heliconiini is crucial to understanding the drivers behind mushroom body expansion in *Heliconius*.

Crucially, the cognitive abilities of *Heliconius* relative to non-pollen-feeding Heliconiini are yet to be experimentally assessed. Experiments do show that *Heliconius* can learn associative colour and shape cues [214,217], yet these abilities are known in other butterflies and presumably widespread amongst Lepidoptera [218–221]. Comparative learning assays across *Heliconius* and non-pollen-feeding Heliconiini are necessary to establish the contexts in which *Heliconius* may be cognitively superior, and whether this can be related to behaviour involved in pollen foraging or the exploitation of host plants.

### 1.9 Potential interactions between life history, diet, behaviour, and neuroanatomy in *Heliconius*

It remains unclear why pollen feeding arose in *Heliconius* but not other butterflies. This question can potentially be answered by combining functional genetics, anatomy, physiology, behaviour, and neuroanatomy. Novel traits often involve considerable costs and may only confer fitness benefits under certain conditions, resulting in unequal landscapes of adaptive opportunity between species [158,174]. Pollen feeding in *Heliconius* is seemingly associated with a constellation of apparently interdependent adaptations including neural elaboration [129,130], increased longevity [132,169], and increased toxicity leading to lower predation rates [222–226] (Figure 1.3). One possibility is that reliable collection of pollen can only be achieved through increased investment in neural tissue and learning, both of which can be costly (Figure 1.3, pink) [54,216,227], but would be favoured by an increase in longevity (Figure 1.3, yellow) [55,56]. However, this interaction may itself require concomitant investment trade-offs or physiological adaptations, as the costs of learning can cause reductions in longevity [228] and fecundity [229]. Strengthened selection for extended lifespans, in turn, seemingly depends on a decrease in adult mortality due to predation, which in *Heliconius* is supported by the aposematic effects of Müllerian mimicry (Figure 1.3, purple) [203,222,223]. While the above hypotheses remain poorly tested, interactions between these diverse traits may be crucial to the cognitive evolution of *Heliconius* butterflies [158].
Figure 1.3 Summary of the hypothesised consequences of increased adult amino acid intake in *Heliconius*, divided into: 1. changes in the allocation of larval resources, green; 2. increased longevity and delayed reproductive senescence, yellow; 3. increases in cyanogenesis, adult distastefulness and aposematism, blue; and 4. increases in behavioural sophistication and neural elaboration, pink. Solid arrows represent amino acid investment, dashed arrows represent selective pressures, and the zig-zag line represents a developmental trade-off. Footnotes indicate supporting evidence for specific traits or relationships.

1.10 Project objectives and methods

The central aim of this project is to understand how cognitive ecology influences brain evolution by investigating mushroom body expansion in *Heliconius* butterflies. Through a comparative framework [45,86], including both *Heliconius* and closely-related Heliconiini, I examine the selective pressures driving this expansion and explore its behavioural consequences. I investigate these questions in three complementary ways:

i. phylogenetic comparative analysis of variation in mushroom body size across the Heliconiini;

ii. comparative behavioural experiments assessing cognition in *Heliconius* and non-*Heliconius* Heliconiini, and;

iii. immunohistochemistry and confocal imaging of Heliconiini brains to investigate possible neural correlates of learning in the mushroom body.
i) Phylogenetic comparative analyses of mushroom body size in Heliconiini butterflies

The independent expansion of a particular region of the brain can provide evidence of natural selection on specific behavioural capacities related to the demands of an animal’s cognitive ecology [17,20]. Data from only three Heliconius species, and two closely related Heliconiini already suggest a dramatic expansion of the mushroom bodies in the Heliconius genus [129,130]. Heliconius are, therefore, potentially a valuable model for investigating the evolutionary relationships between different brain regions, and between brains and behaviour. However, neuroanatomical data on the Heliconiini is limited, coming from only five species (two Heliconius and two non-Heliconius), and crucially there is no data on Eueides, the sister genus of Heliconius. Currently, it is uncertain whether mushroom body expansion is both consistent across the entire genus and limited only to Heliconius, within the Heliconiini.

To build a comprehensive, representative dataset for investigating neuroanatomical variation within the Heliconiini, my project supervisor Stephen Montgomery has collected the brains of 318 Heliconiini individuals from 41 species, including 30 Heliconius. In Chapter 2, I use this dataset to conduct a series of phylogenetic comparative analyses of variation in mushroom body size, along with several other neuropils, across the Heliconiini. These analyses include tests for shifts in the evolutionary rate of particular brain regions, which can be indicative of increased selection coincident with an ecological shift [230]. Ancestral state reconstructions are used to inform where major transitions in mushroom body size are likely to have occurred, as well as the emergence of pollen feeding within the Heliconiini. Finally, I explore several adaptive hypotheses for explaining variation in mushroom body size by testing for associations with ecological traits.

ii) Comparative learning and memory experiments

The likely singular origin of pollen feeding at the base of Heliconius presents a challenge to testing its potential association with mushroom body expansion through phylogenetic analyses of neural traits alone [231]. Comparative behavioural experiments offer a complimentary method for assessing adaptive hypotheses, by directly testing the links between mushroom body expansion and cognitive capacities in extant taxa. In Chapters 3 through 6, I present four different behavioural experiments assessing different cognitive abilities within the Heliconiini, with the aim of testing two main hypotheses explaining mushroom body expansion in Heliconius: (1) the cognitive demands of traplining for pollen, and (2) the identification and learning of host plants based on leaf shape.
In Chapter 3, I assess the spatial learning abilities of *H. melpomene* in a T-maze environment. Conducting comparative studies of Heliconiini spatial learning ability over the large scales at which wild *Heliconius* forage (~1 km²) [133], however, presents several logistical challenges. Nevertheless, traplining seemingly involves several cognitive abilities that are more readily tested in a comparative context across the Heliconiini, including:

i. visual long-term memory,

ii. reversal learning, and;

iii. visual non-elemental learning and memory.

In Chapters 5 and 7, I present comparative behavioural experiments testing these abilities in Heliconiini. Lastly, in Chapter 4, I test whether the expanded mushroom bodies of *Heliconius* are associated with host plant identification and learning by conducting a comparative shape learning experiment.

### iii) Neuroanatomical correlates of learning and memory

In Chapter 6, I investigate the neural correlates of learning within the mushroom body at a microstructural level. The calyx of the mushroom body receives olfactory and visual inputs from the antennal and optic lobes respectively [86], and is comprised of synaptic complexes called microglomeruli [87]. Previous studies have shown that microglomeruli density can vary with both age and experience, and is likely associated with memory formation [88–92]. Furthermore, calyx size similarly varies with age and experience [90,129,215,232]. I investigate whether this age and experience effect differs between *Heliconius* and non-*Heliconius* Heliconiini, which may underpin a potential capacity for improved visual long-term memory in *Heliconius*. To do so, I dissected and imaged brains from *Heliconius erato* and *Dryas iulia* individuals that participated in visual-long term memory experiments, estimating several potential neural correlates of learning and memory: synapse density and total number, Kenyon cell number and calyx volume. Brains from these individuals are compared with age-matched control individuals from a “non-learning” environment, while I also make in-group comparisons testing for correlations between memory performance and the measured neural traits.

Exhibiting an apparent expansion of the mushroom bodies, *Heliconius* promises to be an excellent system for understanding the function and evolution of the mushroom bodies, and
brains more generally. By exploring variation in both the size and underlying neural organisation of the mushroom bodies across the Heliconiini, in combination with behavioural data testing specific adaptive hypotheses, I aim to further our understanding of how brain evolution is shaped by behavioural ecology. As the first study to combine data on *interspecific* variation in mushroom body volume and synaptic connectivity with measures of cognitive performance, this work will offer crucial insight into the evolution of cognitive differences between species.
2 Variation in mushroom body size across Heliconiini butterflies

2.1 Introduction

The evolution of nervous systems is shaped by selective pressures and developmental and functional constraints arising from the structural framework of the brain [36–38,71]. As discussed earlier, the extent to which different regions of the brain can evolve independently is subject to considerable debate [36]. The “concerted” view of brain evolution emphasises the limitation that developmental factors controlling neurogenesis can place on the degree of independence with which separate areas of the brain can evolve [61,62]. The “concerted” model holds that brain evolution is largely shaped by global alterations to the timing of neurogenesis, which changes the sizes of brain components “in concert” [61,62]. The role of selection in determining brain structure is thus reduced in favour of developmental constraints. In contrast, examples of “mosaic” brain evolution show that separate regions of the brain can evolve independently in response to selective pressures, subject to the constraints of functional connectivity between brain regions [17,20,59,60]. The relative importance of “concerted” and “mosaic” evolutionary processes in shaping brain evolution remains a subject of continued debate [63]. Importantly, insects brains provide both examples of developmental constraints and mosaic processes [64–66].

Of particular interest is the evolution of integrative brain centres, such as the cerebral cortex of mammals and the mushroom bodies (MBs) of insects [71]. These regions receive inputs from sensory processing centres and play important roles in cognitive functions such as learning and memory. Mushroom body size and structure varies widely across insects, and has independently undergone volumetric expansion in several distinct groups, including Hymenoptera [21], cockroaches [103,104], beetles [105,106] and Heliconius butterflies [36,130], seemingly in response to divergent selection regimes. This makes the MBs an
interesting case study in understanding the processes and mechanisms that lead to mosaic patterns of brain evolution.

*Heliconius* butterflies, a neotropical genus of approximately 50 species, provide a novel and potentially powerful case study to explore this topic. Data from three *Heliconius* species suggest that mushroom bodies are 3-4 times larger in these taxa than in closely-related Heliconiini, *Dryas iulia* and *Agraulis vanillae* [129,130]. *Heliconius* diverged from their sister genus *Eueides* relatively recently, approximately 18 million years ago, and from other Heliconiini around 26 million years ago, providing the opportunity to investigate the expansion of a higher-order brain region over a relatively small phylogenetic scale. Crucially, there are plausible hypotheses that could explain why mushroom bodies are expanded in *Heliconius*, and existing data on their ecology and phylogenetic relatedness provides the framework to test these hypotheses [131,141,145]. Specifically, changes in the structure of the brain are often associated with behavioural innovation [4,47,73] and *Heliconius* engage in a foraging strategy unique within the Lepidoptera – actively collecting and feeding on pollen [132]. *Heliconius* establish ‘traplines’, routes along which specific plants are regularly visited, suggesting a sophisticated capacity for spatial navigation, likely using learned visual landmarks [133,142,143]. This behaviour is suggestive of enhancements in visually oriented spatial memory and long-term memory retention [133].

The demands of trapline foraging have thus been hypothesised as driving apparent MB expansion in *Heliconius* [129,130]. However, across insects, direct evidence for a functional role of the MBs in spatial memory is limited to evidence from ablation experiments in cockroaches [97] and ants [98,99]. These data are relatively impoverished compared to our understanding of the role of the central complex, another sensory-motor integration structure in the central brain, in insect spatial learning and orientation [210,211]. In fact, visual spatial learning in *Drosophila* does not require the mushroom bodies at all [212]. In addition, an alternative hypothesis has been suggested to explain increases in *Heliconius* MB size, speculatively linking it to host plant use [213]. *Heliconius* lay exclusively on *Passiflora* plants, with varying degrees of specialisation. *Passiflora* display a remarkable diversity of leaf shape, and host plant identification in *Heliconius* appears to be, in part, based on leaf shape recognition and learning through associative conditioning [214]. Mushroom body expansion, may, therefore, support a greater array of search images and enhanced shape-learning abilities, facilitating improved visual identification of host plants [213].
As mentioned before, data on MB size in the Heliconiiini is limited to small numbers of individuals from three *Heliconius* species and two non-*Heliconius* Heliconiini [129,130], and does not include their closest relatives, *Eueides*. This lack of complete data on the variation in MB size within *Heliconius* and across the Heliconiiini currently prohibits proper testing of these adaptive hypotheses. Of particular interest are species from the “Neruda” clade, the only non-pollen-feeding *Heliconius*. These species offer the possibility of decoupling pollen feeding from potentially associated neural elaboration.

Here, I present the first comprehensive phylogenetic comparative analyses of brain structure in the Heliconiiini, sampling 30 species of *Heliconius*, including the “Neruda” species *H. aoede*, and 11 outgroup species, including 5 *Eueides* species. Using immunocytochemistry and confocal imaging, we made volumetric reconstructions of the MB, and other neuropils, including the medulla and antennal lobes which provide visual and olfactory inputs, respectively, to the MBs. We also measured the rest of the central brain for use as an allometric control. I investigate how relative MB size and the scaling relationships between the MB calyx, lobes, and peduncle changes across the Heliconiiini, and within *Heliconius*. I controlled for scaling relationships with other neuropils to test whether MB expansion is solely explained by increased investment in other neuropils, or whether the MBs are expanding independently. I test for variation in evolutionary rate of relative MB size within the Heliconiiini, which may reflect an increase in intensity of natural selection coincident with major ecological shifts, and I also explore whether MB size varies continuously with several ecological traits across the Heliconiiini. Finally, I estimate the ancestral states of MB size and the size of rest of the central brain sizes (rCBR) to identify nodes in the Heliconiiini tree where likely upshifts in relative MB size occurred, in addition to estimating where pollen feeding likely emerged.

### 2.2 Methods

#### i) Neuropil staining and confocal imaging

318 Heliconiiini individuals from 41 species, including 30 *Heliconius*, were collected from wild populations in Panama, Ecuador, Costa Rica, Peru and French Guiana. Individuals were dissected in the field on their day of capture. Individuals were weighed using an OHAUS pocket balance (model YA102), and body length and wingspan were measured using FreeLOGIX digital callipers. Brains were prepared using indirect immunofluorescence staining against synapsin and imaged with a confocal microscope as described in Montgomery,
Merrill & Ott (2016), following Ott (2008) [129,233]. Brains were fixed in zinc-formaldehyde solution and then dissected under HEPES-buffered saline and stored in 100% methanol at -20°C. Brains were then rehydrated in a decreasing methanol series prior to staining with the antisynapsin antibody 3C11 (anti-SYN-ORF1; [234]), followed by applying a Cy2-conjugated antimouse secondary antibody. All brains were imaged with a confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystems, Mannheim, Germany) using a 10X 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 X 512 pixels. The z-step dimension was scaled 1.52X to correct the artifactual shortening associated with the 10X air objective ([235,236]). Acquisition of samples and image data were performed by the project supervisor, Stephen Montgomery.

ii) Neuropil segmentations and volumetric reconstructions

The antennal lobe (AL), the medulla of the optic lobe (ME), the ventral lobula (vLOB), the mushroom body calyx (MBCA), peduncle (MBPED) and lobe (MBLOB), and the rest of the central brain (rCBR) were each segmented to determine their volumes, as described in Montgomery, Merrill & Ott (2016). Using Amira 2020.1, we assigned separate image regions to these structures in the labelfield module by defining outlines based on the brightness of the synapsin immunofluorescence. Every fourth of fifth image in the stack was manually segmented and then interpolated across the z-dimension. Volumes for each neuropil were then calculated using the measure statistics module. As a previous study found no evidence of neuropil asymmetry [129], neuropils were segmented in only one hemisphere and their volumes were then doubled, with the exception of the rest of the central brain which was segmented in its entirety. Image segmentation was performed by me, Daniele Atzeni and the project supervisor, who also checked all segmentations for consistency.

iii) Phylogenetic tree

All phylogenetic comparative analyses were conducted with an unpublished phylogenetic tree of the Heliconiini generated by Francesco Cicconardi, using newly assembled genomes. To assemble the genomes of Heliconius erato cyrbia, H. melpomene rosinia, H. m. amaryllis, and H. cydno Galanthus, Francesco Cicconardi downloaded short-read illumina data from NCBI (National Center for Biotechnology Information) and adopted a reference-guided assembly approach adapted and extended from Lischer and Shimizu (2017) [237]. The strategy involves a first mapping of reads against a reference genome of a related species (H. e. demophoon for H. e. cyrbia; H. m. melpomene 2.5 for H. m. rosinia, H. m. amaryllis and H. cydno for H. cydno...
galanthus) to reduce the complexity of de novo assembly within continuous covered regions, then later integrating reads with no similarity to the related genome.

Extra scaffolding procedures were implemented in order to improve the previous reference guided de novo assembly pipeline [237]. Leveraging on the very small genetic differences of these subspecies with their reference genomes RNA-seq data from these species were downloaded from NCBI concatenated, corrected a normalized using BBMap v 38.79 [238] [target=20 maxdepth=20 mindepth=5]. These reads were mapped using HISAT2 v 2.1.0 [239], and P_RNA_scoffolder [240]. Following this step, RaGOO [241] was used [-T sr], an homology-based scaffolding and misassembly correction pipeline. It identifies structural variants and sequencing gaps, and accurately orders and orients de novo genome assemblies. abyss-Sealer [242] was then used with multiple kmers [-k99 -k97 -k95 -k93 -k91 -k89 -k85 - k81 -k77 -k73 -k69 -k65 -k61 -k57] to finalise the assembly with the attempt to close remaining gaps. After the genome assembly was done, contaminants were identified using BlobTools v 1.1.1 [243] and removed from the final assemblies.

Single-copy orthologous genes in each genome were identified using BUSCO (Benchmarking Universal Single-Copy Orthologs; v3.1.0 [244]). The Insecta set was selected in OrthoDB v 9 (odb9 1958 genes) using default parameters in genome [-m genome]. For genes recovered as fragmented and missing a further mapping was implemented mapping with Exonerate using protein sequences found in other Heliconius species to deal with possible false discovery hits. For each species, all BUSCO genes found in a single copy were used for phylogenetic analysis.

Each nucleotide sequence BUSCO locus was aligned separately with MACSE v 2 [245], and all alignment concatenated. From the concatenated alignment gaps were removed using Gblock v 0.91b [246] [-t=c -b1=(#Nseq/2+1) -b2=(#Nseq/2+1) -b3=1 -b4=6 -b5=h] following [247,248]. A maximum likelihood (ML) phylogenetic tree was performed using IQ-TREE v 2 [249], partitioning the supermatrix for each locus and codon position. IQ-TREE was run with the following settings: --runs 5 -m MFP. 5,000 ultrafast bootstrap replicates were conducted, resampling partitions and then sites within resampled partitions [250,251]; a strategy that may help to reduce false positives (-b 5000 --sampling GENESITE).

The Bayesian algorithm of MCMCTree [252] was implemented with approximate likelihood computation to estimate divergence times. First, branch length was estimated by ML and then the gradient and Hessian matrix around these ML estimates in MCMCTree using the
DNA supermatrix. Calibration nodes were constrained according to Cicconardi et al. (*in prep*), using a uniform distribution. To ensure convergence, the analysis was run for $10 \times 100k$ generations after a 10M generations as burn-in, logging every 200 generations. *Tracer* v 1.7.1 [253] was used to check for convergence and ESS values were $> 200$.

**iv) Phylogenetic comparative analyses**

All volumetric measurements were log$_{10}$-transformed for statistical analyses. Where possible, analyses were conducted using individual-level data, though certain analyses required the use of species averages, calculated as the arithmetic mean. All statistical analyses were performed in R v 4.1.2.

I ran a series of phylogenetic generalised linear mixed models (GLMM) with gaussian distributions using the R package *MCMCglmm* v 2.32 [254] to determine whether the scaling relationship between the MB and the rest of the central brain (rCBR) changes between *Heliconius* and non-*Heliconius* Heliconiini, and between Heliconiini genera and subclades within *Heliconius* [255]. I ran a series of models that variously included the effects of body size measurements and sex. I also included antennal lobe (AL) and medulla (ME) volumes to test whether increases in sensory neuropils, and hence increased sensory inputs, could, alone, explain variation in MB size. The best fitting model was then identified according to the Deviance Information Criterion [256]. To determine whether any increases in MB size are driven by expansion in a particular region of the MB, I also tested for variation in the scaling relationship between the mushroom body calyx and the lobe and peduncle between Heliconius and non-*Heliconius* species. I also assessed whether the MB are unique in exhibiting expansion in *Heliconius* by testing whether the genus shows an increase in ME or AL size, controlling for rCBR. Additionally, I tested for variation in rCBR size, using body size measurements as an allometric control. Finally, I explored whether certain ecological factors could explain MB variation. To do so, I took the best fitting model explaining variation in MB size and then included two ecological factors – the degree of social roosting, and pollen feeding (which was tested by removing the *Heliconius* factor with which it is almost perfectly confounded). All models were checked for convergence using the *gelman.diag* function and for auto-correlation using the *autocorr* function provided in *MCMCglmm*, in addition to visually inspecting the trace plots. All models were run for 500,000 iterations, with a burn-in of 10,000 and a thinning factor of 500.
To support these analyses, I performed two additional sets of analyses to examine allometric shifts between MB size and rCBR. First, the function *sma* from the R package *smatr* v 3.4-8 was used to test for pairwise interspecific differences in the scaling relationship between MB volume and rCBR across all species, testing for both differences in the slope and elevation of scaling relationships [257]. For this analysis, only species with at least 8 individuals were included, reducing the dataset to 26 species, and the “robust” option was set to true for these analyses [258]. Second, the R package *bayou* v 2.0 was used to identify regions of the Heliconiini tree showing evidence of a shift in the scaling relationship between MB volume and rCBR [259]. This method fits multi-optima Ornstein-Uhlenbeck models to phylogenetic comparative data, estimating the placement and magnitude of adaptive shifts. I compared three models: one with no shifts in either slope or elevation (all species are assumed to exhibit the same allometric scaling relationship), one allowing for shifts in elevation (a common allometric slope is assumed, but “grade-shifts” are allowed), and one allowing for shifts in both elevation and slope. Models were each run for 1,000,000 iterations, with a burn-in of 300,000 generations and their fits were compared for fit by using the *steppingstone* function in *bayou* and running it for 10,000 generations with a burn-in of 3000 [260]. The posterior probability cut-off for identifying a shift in the relationship between MB and rCBR size was set at 0.3 [259,261,262].

To identify key periods of evolutionary change in a robust fashion, I used two different methods to test for shifts in the evolutionary rate of change in MB size within the Heliconiini. Firstly, I used the program *BayesTraits* v 3 to compare two independent contrast MCMC models of evolution, one allowing for a rate scaling parameter to vary across branches, and a second one where it is averaged across all branches [263,264]. In the variable rate model, branch lengths were scaled to accommodate periods of increased trait change, and this scaling parameter provides an indication of evolutionary rate. Models were run for 110,000,000 iterations, with a burn-in of 10,000,000, and sampled every 10,000 iterations. Estimated marginal likelihoods for each model were calculated using the stepping stone sampler [265], sampling 100 stones for 10,000 iterations each. I compared the variable rates model with the non-variable rates model by calculating the Log Bayes Factor.

Secondly, I used a recently-published method that uses Brownian motion to model variations in evolutionary rate [266]. Under this model, different branches of the tree are assumed to have different evolutionary rates, which also evolve via a Brownian process. Unlike the Bayesian approach implemented in *BayesTraits*, this method involves continuous (rather than discrete) changes in evolutionary rate between, or along, branches. This method has been
implemented in the `multirateBM` function in the R package `phytools` v 0.7-90 [267]. I used this method to estimate variations in the evolutionary rate of MB size, rCBR size and the residuals calculated from a phylogenetic regression of MB size against rCBR size using the `phyl.resid` function in `phytools`.

I used two methods to estimate MB size and rCBR size at key internal nodes within the Heliconiini tree. Firstly, using `BayesTraits` v 3 I compared a random walk and directional MCMC models of evolution in MB size, controlling for rCBR, and using the series of scaled trees generated during the iterations of the variable rates model described above. The Log Bayes Factor (-16.84) for these two models indicated that a directional model of evolution was not supported. I then used the non-directional model and the scaled trees to estimate values for MB size and rCBR size at key internal nodes. Secondly, I used the `fastAnc` function in the `phytools` package to estimate the maximum likelihood ancestral states for MB size and rCBR size at each node in the Heliconiini tree.

The state of the discrete trait of pollen feeding at internal nodes was estimated using three different methods: (1) MCMC stochastic character mapping in `phytools` for 1000 simulations, (2) maximum likelihood using the `ace` function in the R package `ape` v 5.5 [268], and (3) maximum parsimony using the `asr_max_parsimony` function in the R package `castor` v 1.7.0 [269]. The `fitDiscr` function from the R package `geiger` 2.0.7[270] was used to determine the best fitting transition model between equal rates, symmetric and all rates differ. Equal rates was the best fitting model and was used for all downstream analyses.

### 2.3 Results

**i) Variation in mushroom body size across the Heliconiini**

The best phylogenetic generalised linear mixed model testing for a difference between MB size in *Heliconius* and non-*Heliconius* Heliconiini, included the rest of the central brain (rCBR), antennal lobes (AL) and medulla (ME) volumes as independent variables, as well as an interaction term between sex and membership in the *Heliconius* genus (Table 2.1, DIC -893.7593). Across the Heliconiini, MB size varies significantly with rCBR, AL and ME volumes (Table 2.1), suggesting some allometric effects of whole brain size, and individual effects of size variation in sensory neuropils that project to the MB. However, even when controlling for these effects, MB size in *Heliconius* individuals is significantly higher than in
other Heliconiini (Table 2.1, Figure 2.1(a)). There was also a significant interaction between sex and Heliconius, with Heliconius females displaying significantly larger mushroom bodies, with no similar effect seen in the outgroup Heliconiini (Table 2.1, Figure 2.2). When controlling for rCBR, none of body length, wingspan or body mass had any significant explanatory relationship with MB size. Unlike the MBs, relative AL (pMCMC=0.186) and ME (pMCMC=0.480) were not significantly larger in Heliconius, when controlling for rCBR. In addition, rCBR volumes were not higher in Heliconius when controlling for either wingspan (pMCMC=0.276), body length (pMCMC=0.457) or body mass (pMCMC=0.237). The scaling relationship between the mushroom body calyx and the lobes and peduncle does not differ between Heliconius and the outgroup Heliconiini (pMCMC=0.180). Indeed, it is remarkably conserved, suggesting these regions of the MB increased in size in concert, mostly likely with a common circuit architecture (Figure 2.3).

Including phylogenetic grouping (outgroup genus or subclade within Heliconius) as a factor reveals that MB size, controlling for rCBR, AL and ME, is internally consistent both within Heliconius and amongst the outgroup Heliconiini (Table 2.2). However, MB size in Dryadula phaetusa is not significantly different to either the Heliconius clades or the other outgroups, while Eueides only differ significantly from the melpomene-group and Silvaniform Heliconius (Figure 1.1(g), Table 2.2). Uncorrected pairwise comparisons, however, do show Dryadula phaetusa and Eueides as having significantly larger MBs than several outgroup Heliconiini, and smaller MBs than some Heliconius (Table 2.2).

Table 2.1 Summary of results for phylogenetic generalised linear model testing for the effect of membership in the Heliconius genus on mushroom body size, controlling for the volumes of the rest of the central brain, antennal lobe, and medulla and a sex interaction with Heliconius.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Posterior mean</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
<th>pMCMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest of central brain volume</td>
<td>0.475</td>
<td>0.330</td>
<td>0.640</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Antennal lobe volume</td>
<td>0.328</td>
<td>0.202</td>
<td>0.436</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Medulla volume</td>
<td>0.277</td>
<td>0.111</td>
<td>0.453</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Heliconius</td>
<td>0.457</td>
<td>0.280</td>
<td>0.654</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Heliconius0:Sex</td>
<td>0.00573</td>
<td>-0.0217</td>
<td>0.0373</td>
<td>0.629</td>
</tr>
<tr>
<td>Heliconius1:Sex</td>
<td>-0.0452</td>
<td>-0.0604</td>
<td>-0.0274</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>
Chapter 2 – Variation in mushroom body size across Heliconiini butterflies

Figure 2.1 (a) Allometric grade-shifts in MB size across the Heliconiini, with groups and regression lines based on elevation shifts identified, holding slope constant, with a posterior probability greater than 0.3 using the R package bayou (red = Heliconius spp.; yellow = Eueides spp.; purple = Dryadula phaetusa; blue = remaining outgroup Heliconiini). Each data point represents one individual. (b) Areas of the Heliconiini tree where a grade-shift in the relationship between MB and rCBR size is identified with a posterior probability greater than 0.3, allowing for variations in elevation, but not slope. Size of green circles represents the posterior probability. (No. individuals = 318, no. species = 41 (30 Heliconius)).
Figure 2.2 Mushroom body volume plotted against the volume of the rest of the central brain (rCBR) for *Heliconius* individuals (n=243), grouped by sex. In *Heliconius* females have significantly larger mushroom bodies when controlling for the volumes of the rCBR, the medulla, and the antennal lobes (Table 1). Dashed lines show phylogenetic linear regressions for each sex in *Heliconius*.

Figure 2.3 MBCA volume plotted against combined volumes of MBLOB and MBPED. The scaling relationship between these two volumes is not different between *Heliconius* (red) and the non-*Heliconius* Heliconini (other colours). Colour groupings based on bayou results shown in Figure 2.1. Red = *Heliconius*; yellow = *Eueides*; purple = *Dryadula phaetusa*; blue = other outgroup Heliconini. Dashed line shows linear regression between the two traits (t=93.837, d.f.=316, P<2e-16, R²=0.965). MBCA = mushroom body calyx; MBLOB = mushroom body lobe; MBPED = mushroom body peduncle.
Table 2.2 Pairwise comparisons between Heliconini clades for mushroom body size, derived from a phylogenetic generalised linear mixed model including the size of the antennal lobe, the medulla and the rest of the central brain as fixed effects. P-values above the diagonal are uncorrected, and below are corrected for multiple comparisons using Tukey’s test. Heliconius clades shown in Figure 1.1(g). P-values < 0.001 indicated by “***”, < 0.01 by “**”, < 0.05 by “*” and < 0.1 by “.”.

<table>
<thead>
<tr>
<th></th>
<th>Non-Heliconius Heliconini</th>
<th>Heliconius clade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Philaethria</td>
<td>Dryadula</td>
</tr>
<tr>
<td>No. Species</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No. individuals</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Philaethria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dryadula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Podotricha</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dryas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dione</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agraulis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eueides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>erato</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>sara/sapho</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>aeode</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>doris</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>wallacei</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Silvaniforms</td>
<td>***</td>
<td>**</td>
</tr>
<tr>
<td>melpomene</td>
<td>***</td>
<td>**</td>
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</table>
To further identify periods of independent MB evolution I interrogated scaling relationships with rCBR across Heliconiini. Of the three bayou models tested estimating the evolution of MB and rCBR, the model allowing for shifts in elevation, but not slope (marginal likelihood = 51.080), was better supported than both the model allowing for shifts in elevation and slope (marginal likelihood = 30.000) and the model allowing for no shifts (marginal likelihood = 25.112). This model identified four shifts with a posterior probability greater than 0.3 (Figure 1(b)). The model estimates with a posterior probability of 0.64 an increase in MB size at the base of Heliconius and Eueides, followed by an additional increase at the base of Heliconius with a posterior probability of 0.66, and a decrease at the base of Eueides with a posterior probability of 0.71. An increase in MB size along the branch leading to Dryadula phaetusa is also identified with a posterior probability of 0.98.

Supporting this result, pairwise allometric analysis with the R package smatr indicates that the slope of the allometric relationship between rCBR and MB size does not significantly differ between Heliconiini species. However, there are significant interspecific differences in elevation (Table 2.3). In general, relative MB size is consistent throughout Heliconius and larger than in the outgroups, consistent with the results of the phylogenetic GLMMs above. Eueides and Dryadula phaetusa are revealed to have relative MB sizes intermediate between Heliconius and the other outgroups, while H. telesephe, H. doris and H. aoede have significantly smaller MBs than several other Heliconius species.
**Table 2.3** Interspecific differences in elevation in the relationship between the size of the mushroom bodies and the rest of the central brain. P-values above the diagonal are uncorrected, and below are corrected for multiple comparisons. P-values < 0.001 indicated by “***”, < 0.01 by “**”, < 0.05 by “*” and < 0.1 by “.”. *Avan* = *Agraulis vanillae*; *Dial* = *Dryas iulia*; *Dpha* = *Dryadula phaetusa*; *Eisa* = *Eueides Isabella*; *Evib* = *Eueides vibilia*; *Haoe* = *H. aoede*; *Hcyd* = *H. cyndo chioneus*; *Hcyg* = *H. cyndo galanthus*; *Hdor* = *H. doris*; *Herc* = *H. erato cyrbia*; *Hhel* = *H. helocharis*; *Hhis* = *H. himera*; *Hism* = *H. ismenius*; *Hmer* = *H. melpomene amaryllis*; *Hmex* = *H. melpomene melipomene*; *Hmer* = *H. melpomene rosina*; *Hnum* = *H. numata*; *Hpac* = *H. pachinus*; *Hsap* = *H. sapho*; *Hsar* = *H. sara*; *Htel* = *H. telesiphe*; *Htim* = *H. timareta*; *Hwal* = *H. wallacei*; *Pdid* = *Philaethria dido*.

<table>
<thead>
<tr>
<th>Heliconius</th>
<th>Non-Heliconius Heliconiini</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hpac</strong></td>
<td><strong>Evib</strong></td>
</tr>
<tr>
<td><strong>Hcyg</strong></td>
<td><strong>Eisa</strong></td>
</tr>
<tr>
<td><strong>Hcyd</strong></td>
<td><strong>Avan</strong></td>
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<tr>
<td><strong>Htim</strong></td>
<td><strong>Dial</strong></td>
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<tr>
<td><strong>Hmer</strong></td>
<td><strong>Dpha</strong></td>
</tr>
<tr>
<td><strong>Hmea</strong></td>
<td><strong>Pdid</strong></td>
</tr>
<tr>
<td><strong>Hhim</strong></td>
<td><strong>Eisa</strong></td>
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<td><strong>Hism</strong></td>
<td><strong>Avan</strong></td>
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<td><strong>Hnum</strong></td>
<td><strong>Dial</strong></td>
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<td><strong>Hwal</strong></td>
<td><strong>Dpha</strong></td>
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<td><strong>Hdor</strong></td>
<td><strong>Pdid</strong></td>
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<tr>
<td><strong>Haoe</strong></td>
<td><strong>Eisa</strong></td>
</tr>
<tr>
<td><strong>Hsap</strong></td>
<td><strong>Avan</strong></td>
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<td><strong>Hsar</strong></td>
<td><strong>Dial</strong></td>
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<tr>
<td><strong>Herc</strong></td>
<td><strong>Dpha</strong></td>
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<tr>
<td><strong>Hhel</strong></td>
<td><strong>Pdid</strong></td>
</tr>
<tr>
<td><strong>Hhim</strong></td>
<td><strong>Eisa</strong></td>
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<tr>
<td><strong>Htim</strong></td>
<td><strong>Avan</strong></td>
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<td><strong>Hwal</strong></td>
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<td><strong>Hdor</strong></td>
<td><strong>Dpha</strong></td>
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<td><strong>Haoe</strong></td>
<td><strong>Pdid</strong></td>
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<td><strong>Hsar</strong></td>
<td><strong>Avan</strong></td>
</tr>
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<td><strong>Herc</strong></td>
<td><strong>Dial</strong></td>
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<td><strong>Hhel</strong></td>
<td><strong>Dpha</strong></td>
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<tr>
<td><strong>Htim</strong></td>
<td><strong>Pdid</strong></td>
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<tr>
<td><strong>Hwal</strong></td>
<td><strong>Avan</strong></td>
</tr>
<tr>
<td><strong>Hdor</strong></td>
<td><strong>Dial</strong></td>
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<tr>
<td><strong>Haoe</strong></td>
<td><strong>Dpha</strong></td>
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<tr>
<td><strong>Hsar</strong></td>
<td><strong>Pdid</strong></td>
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<tr>
<td><strong>Herc</strong></td>
<td><strong>Avan</strong></td>
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<tr>
<td><strong>Htim</strong></td>
<td><strong>Dpha</strong></td>
</tr>
<tr>
<td><strong>Hwal</strong></td>
<td><strong>Pdid</strong></td>
</tr>
</tbody>
</table>

* Interspecific differences in elevation in the relationship between the size of the mushroom bodies and the rest of the central brain.

- **Avan** = *Agraulis vanillae*
- **Dial** = *Dryas iulia*
- **Dpha** = *Dryadula phaetusa*
- **Eisa** = *Eueides Isabella*
- **Evib** = *Eueides vibilia*
- **Haoe** = *H. aoede*
- **Hcyd** = *H. cyndo chioneus*
- **Hcyg** = *H. cyndo galanthus*
- **Hdor** = *H. doris*
- **Herc** = *H. erato cyrbia*
- **Hhel** = *H. helocharis*
- **Hhis** = *H. himera*
- **Hism** = *H. ismenius*
- **Hmer** = *H. melpomene amaryllis*
- **Hmea** = *H. melpomene melipomene*
- **Hmer** = *H. melpomene rosina*
- **Hnum** = *H. numata*
- **Hpac** = *H. pachinus*
- **Hsap** = *H. sapho*
- **Hsar** = *H. sara*
- **Htel** = *H. telesiphe*
- **Htim** = *H. timareta*
- **Hwal** = *H. wallacei*
- **Pdid** = *Philaethria dido*
ii) Rate of evolution of mushroom body size varies across the Heliconiini tree

Pronounced shifts in MB scaling suggest there have been periods of evolution where MB size expanded rapidly. Indeed, results from BayesTraits show support for a variable rates model of evolution for MB size, controlling for rCBR (Log Bayes Factor = 14.556). Under this model, the branch leading to Heliconius has the second largest mean scalar (8.238), while the branch leading to H. clysonymous and H. telesephe has the largest mean scalar (9.038, Figure 2.4). Conversely, the branches at the base of Eueides (1.477) and Eueides + Heliconius (1.322) are calculated as having much lower scaling factors (Figure 2.4).

The phytools Brownian motion method for modelling change in evolutionary rate, my independent method to test for consistency in the branches identified as showing elevated rates of evolution by BayesTraits shows that the highest rates of evolution in MB size were estimated along the branch leading to Heliconius (Figure 2.5(a)), which did not show similarly elevated rates of rCBR size (Figure 2.5 (b)-(c)). The branches leading to Heliconius + Eueides and to Eueides, also show high rates of MB size evolution, relative to rCBR, though less dramatically than in the branch leading to Heliconius. Dryadula, similarly shows high rates of evolution in MB size, relative to rates of rCBR evolution. Modelling the size-corrected residuals of MB size, calculated by running a phylogenetic regression against rCBR size, estimates that highest evolutionary rates as instead occuring at the base of Heliconius and Eueides, with both the branches leading to Heliconius and Eueides then experiencing further periods of elevated evolutionary rate (Figure 2.5(d)).

These large jumps in relative MB size are reflected in the estimated ancestral states for MB and rCBR volumes within the Heliconiini tree (Figure 2.6(a)). Both the BayesTraits MCMC random walk method and the phytools maximum likelihood methods place the root of the Heliconiini tree as having MBs slightly larger than the extant outgroups (Figure 2.6(a), blue). An increase in MB size is then estimated along the branch leading to Heliconius and Eueides (Figure 2.6(a), orange), before an even greater expansion at the base of Heliconius (Figure 2.6(a), red). The two methods are generally consistent in their estimation of the ancestral states at these nodes. However, the phytools maximum likelihood method estimates a greater increase in MB size at the base of Heliconius and Eueides, compared with the MCMC random walk of run using BayesTraits.
Figure 2.4 (a) Consensus scaled tree produced from multiple iterations of a variable rates model of the evolution of mushroom body size (controlling for the size of the rest of the central brain) in BayesTraits, generated using an originally ultrametric tree. Increased branch lengths indicate an estimated increase in the evolutionary rate of MB size. (b) Scaled branch lengths, calculated using mean scalars, plotted against original branch length, with highly scaled branches labelled. Dashed line shows $y = x$. Ma = million years ago.
Figure 2.5 Estimated evolutionary rates of (a) mushroom body (MB) volume, and (b) rest of central brain (rCBR) volume in the Heliconiini, modelled with Brownian Motion. (c) Estimated evolutionary rates of MB size plotted against estimated evolutionary rates of rCBR size, with key tree branches labelled. Dashed line indicates y=x. (d) Estimated of evolutionary rates of residual MB size, corrected for rCBR size.
iii) Ecological predictors of mushroom body size

Finally, I tested whether certain ecological factors explained variation in MB size. The degree of social roosting had no effect on MB size (pMCMC=0.794). However, including pollen feeding as a factor instead of *Heliconius* showed a similarly good fit, with consistent results (Table 2.4, DIC: -895.6749). However, pollen feeding is present in all *Heliconius* in the dataset, except for the “Neruda” species, *H. aeode*, and absent in all outgroup Heliconiini. Both MCMC stochastic character mapping and maximum likelihood estimations indicate pollen feeding
almost certainly arose only once in the Heliconini, at the base of Heliconius, and was secondarily lost in the ‘Neruda’ clade, which includes *H. aeode* (Figure 2.6(b)-(c)). Alternatively, maximum parsimony indicates that it is equally likely that pollen feeding arose once and was lost once or arose twice within *Heliconius* (Figure 2.6(d)). This presents a difficulty to estimating the effect of pollen feeding on MB size, independent of the *Heliconius* effect. Importantly, *H. aeode*, do not exhibit a reduction in MB size, falling within the range of other, pollen-feeding *Heliconius* species (Tables 2.2 & 2.3).

**Table 2.4** Summary of results for a phylogenetic GLMM testing for the effect of the presence of pollen feeding, including an interaction with sex, on mushroom body volume, controlling for the volumes of the rest of the central brain, antennal lobe, medulla.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Posterior mean</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
<th>pMCMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest of the central brain volume</td>
<td>0.475</td>
<td>0.306</td>
<td>0.630</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Antennal lobe volume</td>
<td>0.322</td>
<td>0.219</td>
<td>0.447</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Medulla volume</td>
<td>0.288</td>
<td>0.124</td>
<td>0.469</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Pollen feeding</td>
<td>0.249</td>
<td>0.0899</td>
<td>0.408</td>
<td>0.004**</td>
</tr>
<tr>
<td>Pollen feeding0:Sex</td>
<td>0.00595</td>
<td>-0.0209</td>
<td>0.0322</td>
<td>0.623</td>
</tr>
<tr>
<td>Pollen feeding1:Sex</td>
<td>-0.0482</td>
<td>-0.0645</td>
<td>-0.0302</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

### 2.4 Discussion

I present here the first phylogenetic comparative analyses of the volumetric relationships between different brain regions across Heliconini butterflies. My analyses show that, controlling for the volume of the rest of the central brain (rCBR), the elaboration of the mushroom bodies (MBs), previously described in only a few species of *Heliconius* [129,130], is present across the entire genus. This is the case even when additionally controlling for AL and ME, regions providing olfactory and visual information to the MBs, suggesting that this expansion is not simply a result of increased input. In this way, *Heliconius* exhibit a convergence with several other insect groups including parasitoid and parasitic wasps [21], cockroaches [103,104], generalist scarab beetles [105,106], dragonflies and damselflies [93] and termites [271] which also show expanded MBs.

More unexpectedly, I detected several upshifts in MB size within the wider Heliconini, in addition to the expansion within *Heliconius*. Firstly, several analyses resolve the *Eueides* spp. as having larger relative MBs than the other outgroup Heliconini, though smaller than *Heliconius* (Figure 1, Tables 2.2 & 2.3). Ancestral state reconstruction and Bayesian reversible-
jump multi-optima OU modelling indicate that a marked increase in MB occurred along the branch leading to *Heliconius* + *Eueides*, followed by either a conservation of this increased MB size in *Eueides*, or a slight decrease in MB size along the *Eueides* branch. Secondly, *Dryadula phaetusa* appears to have also independently experienced an increase in MB size (Figure 2.1), having MBs comparable in size with *Eueides*. Within *Heliconius*, relative MB size appears more conserved, although there is evidence of some variation within the genus. For example, *H. doris*, *H. wallacei* and the non-pollen-feeding *H. aoede* stand out as having relatively smaller MBs than most other *Heliconius* (Tables 2.2 & 2.3).

Importantly, within the Heliconiini, MB size appears to have evolved independently of other brain regions as neither the AL, ME or vLOB show a *Heliconius*-specific increase in relative size. Similarly, rCBR itself, a proxy for overall brain size, is not larger in *Heliconius*, when controlling for body size measurements. The expansion of the MBs in *Heliconius* therefore seems to represent the independent elaboration of a specific region of the brain, reflecting a mosaic pattern of brain evolution [20,36]. In other groups, such mosaic expansion of an individual brain region has been interpreted as an adaptation in response to specific ecological pressures [20,60,66,272–275]. Indeed, these results show evidence of increased evolutionary rates of relative MB size, most notably along the branch leading to the last common ancestor of *Heliconius* and *Eueides*, and along the branch leading to *Heliconius* (Figures 2.4 & 2.5). Notably, *Heliconius* diverged from *Eueides* relatively recently, approximately 18 million years ago, and aside from pollen feeding, exhibit close ecological similarity to the other Heliconiini, making them an excellent system for investigating adaptive hypothesis relating to brain expansion.

Two main adaptive hypotheses have been proposed to explain MB expansion within the Heliconiini. The first suggests that it is associated with the increased cognitive demands of trampine foraging for pollen by *Heliconius* butterflies [129,130], and the second that it was driven by the learning and recognition of host plants [213]. I found no association between the number of host plants used, and relative MB size across the Heliconiini. However, this hypothesis could be explored in greater depth in further studies by assessing the shape learning abilities of the Heliconiini, and by testing for associations between MB size and the morphospace of leaf shapes exploited, rather than simple the number of host plants (see Chapter 4). In contrast, a major period of MB expansion coincided with the origin of pollen feeding, and the presence of pollen feeding was significantly associated with larger MBs (Table 2.4). This could be consistent with MB expansion in *Heliconius* being driven by the behavioural
innovation of pollen feeding, however, it is difficult to disentangle a general genus effect of *Heliconius* from pollen feeding per se. Furthermore, the non-pollen-feeding *H. aoede* does not show a reduction in MB size, falling within the range of other pollen-feeding *Heliconius* species (Tables 2.2 & 2.3). Any explanation of MB expansion within *Heliconius* must also account for MB expansion along the *Heliconius-Eueides* branch, as well as in *Dryadula phaetusa*, neither of which co-occurs with pollen feeding behaviour. Clearly, pollen feeding alone cannot explain all the variation in relative MB size throughout the Heliconiini. It is possible that certain behaviours emerged at the base of *Heliconius* and *Eueides* providing a foundation for the later emergence of pollen feeding. Generally, *Heliconius* collect pollen predominantly from cucurbitaceous vines, which serve as a dependable but relatively rare resource [133,137–139]. Spatially faithful foraging on these plants appears to depend on the maintenance of a stable home range and roost-site fidelity. Indeed, *Heliconius* establish long-term, stable home ranges, within which individuals regularly return to the same roosting locations [143,197]. Unfortunately, there is little data on the extent to which other Heliconiini establish long-term, stable home ranges. Interestingly, however, marked territorial behaviour has been observed in *Eueides tales* and *Eueides aliphera* [276]. One possibility is that territorial behaviour and stable home ranges, which presumably depend on enhanced spatial learning abilities [134], emerged at the base of *Heliconius* and *Eueides*, driving MB expansion along that branch. This may have then facilitated the emergence of pollen feeding which selected for further MB expansion in the branch leading to *Heliconius*. However, I currently lack the behavioural data to formally test this hypothesis. Similarly, the lack of data on *Dryadula phaetusa* behaviour in the wild make it difficult to constructively speculate on the selective pressures driving MB expansion in that species.

Despite these other instances of MB expansion in the Heliconiini, the distinct expansion event at the base of *Heliconius*, coupled with a likely increased evolutionary rate, is consistent with a role of pollen feeding driving this neural elaboration. Notably, within *Heliconius*, females had larger MBs than males, an effect not seen in the other Heliconiini (Table 2.1, Figure 2.2). *Heliconius* females generally collect more pollen than males [138,169], and the increased MB size in females may be reflective of an increased investment in pollen collection.

The likely singular origin of pollen feeding at the base of *Heliconius* (Figure 2.6(b), (c)) presents a challenge to testing its potential association with MB expansion through phylogenetic analyses of neural traits alone. Comparative behavioural data offers one way of better directly testing the link between MB expansion and the cognitive demands of trapline
foraging for pollen. However, the cognitive abilities of *Heliconius* relative to non-pollen-feeding Heliconiini are yet to be experimentally assessed. Testing the cognitive abilities presumably required for trapline foraging, such as enhanced spatial memory and long-term memory retention, is necessary to properly test the role of pollen feeding in driving the MB expansion of *Heliconius* (Chapters 3, 5 & 7). Such behavioural data could provide key evidence regarding the functional consequences of MB expansion, which growing data suggests can vary in response to different selective pressures [86], and particularly inform the role of the MBs in spatial learning for which only limited data currently exists [97–99].

The results described here also position *Heliconius* as an excellent model system for investigating the genetic and developmental basis of region-specific neural elaboration. It has been argued that the convergent elaboration of the MBs in several different groups of insects reflects a limited set of developmental mechanisms controlling their expansion [71]. Determining the genetic and developmental underpinnings of MB expansion in *Heliconius* could therefore offer crucial insight into the mechanisms governing brain evolution, particularly given the relatively small phylogenetic distances between Heliconiini species. One clue may be provided by the fact that the relative volumes of the MBCA and the MBPED and MBLOB do not vary across the Heliconiini, suggesting that MB expansion in *Heliconius* is driven by a replication of existing neural circuitry, rather than an increase in a specific region of the MB.

In summary, these results reveal a distinct and dramatic expansion of the MBs in *Heliconius*, in what appears to be a clear example of the mosaic evolution of a specific brain region, which seemingly began along the branch leading to *Heliconius* and *Eueides*. Although the expansion of the MB in *Heliconius* is partly consistent with having been driven by pollen feeding, the increase in MB size along the *Heliconius-Eueides* branch predates the evolution of pollen feeding, suggesting that other unexplored selective pressures are also shaping MB evolution. However, the close phylogenetic relatedness of the Heliconiini, and considerable associated genetic resources [145], position the clade as an excellent system as for investigating the selective pressures neural elaboration and the genetic and developmental mechanisms that underpin it.
3 Spatial learning in a T-maze by the butterfly *Heliconius melpomene*

3.1 Introduction

The mushroom bodies are a structure of the insect brain that are important for associative learning and memory. Growing evidence suggests that mushroom body function varies across insect groups in response to different selective pressures [86]. *Heliconius* butterflies, a neotropical genus of approximately 50 species, exhibit a marked expansion of the mushroom bodies, which are 3-4 times larger than in other Lepidoptera, including closely related Heliconiini genera (Chapter 2) [129,130]. The dramatic expansion of mushroom bodies in *Heliconius* butterflies makes them an ideal system for investigating the behavioural consequences of mushroom body expansion. However, there is currently very little published data on the comparative cognitive abilities of Heliconiini butterflies [277] and the selective pressures driving this expansion remain unknown.

As discussed in Chapter 1, the primary hypothesis as to why mushroom bodies are so dramatically expanded in *Heliconius* relates to their novel foraging behaviour – traplining for pollen [129,130,134]. Traplining is a foraging strategy in which an animal repeatedly returns to food sources, following a specific route [278]. Generally, *Heliconius* collect pollen from sparse, but reliable, *Psiguria* and *Gurania* vines, following foraging routes along which specific plants are visited with a high degree of spatial and temporal regularity [133,142,143]. *Heliconius* traplines centre on a limited home range of 100 m$^2$ to 1 km$^2$ and return to the same roosting locations at night, apparently located using visual cues [143,197,203]. These observations suggest *Heliconius* possess a sophisticated capacity for navigation using learned visual landmarks, similar to behaviours observed in certain bees [201,202].

Currently, our knowledge of *Heliconius* foraging behaviours and roost site fidelity comes exclusively from field observation studies. Mark-release-recapture studies, where wild individuals are caught and marked, have shown *Heliconius* individuals to repeatedly return to the same pollen sources over successive days, with a high degree of temporal consistency.
Movement patterns have been reconstructed from these data showing individuals to be following consistent foraging routes along which one or more pollen sources are visited. Wild *Heliconius* have been observed to maintain consistent traplines for up to 90 days and generally return to the same roosting site at night [143]. Importantly, traplining routes are highly individualistic and differ markedly between butterflies that share a roosting site, suggesting that memory is guiding their movements, rather than individuals simply responding to environmental stimuli [143]. The mechanism by which *Heliconius* navigate remains unknown, however, a reliance on visual landmarks seems plausible. Consistent with this, experimental displacement of roosting branches resulted in individuals returning to the original roosting site, rather than seeking out the displaced branches [143,279]. However, many assumptions about their spatial memory remain untested experimentally. A recent study shows that experimentally translocated *Heliconius* quickly orientate towards, and return to, their site of origin after release [205]. An unpublished experiment, however, failed to find any evidence of *Heliconius* spatial learning in a 61 x 61 cm grid [280], indicating that *Heliconius* may only learn spatial information at larger scales. There is, therefore, a need to experimentally validate the presumed ability of *Heliconius* butterflies to learn spatial information in a manner more closely approximating their large-scale foraging behaviours.

In traplining, *Heliconius* exhibit a foraging behaviour unknown in other Lepidoptera, with the possible exception of *Jalmenus evagoras*, where males have tentatively been described as traplining for female pupae [281]. Traplining, relies on the ability to learn the locations of both roosting sites and food sources, suggesting a capacity for spatial learning remarkable amongst Lepidoptera. Although the mechanisms of navigation used by *Heliconius* are unknown, traplining presumably depends on the ability to integrate complex information relating to resource locations (likely visual cues) and maintain these memories over extended periods of time [143]. Traplining, therefore, appears to be a far more cognitively demanding foraging strategy than the opportunistic behaviours observed in other Lepidoptera, which are not known to exploit specific food sources with spatial regularity.

The apparent cognitive demands of trapline foraging are hypothesised to have driven mushroom body expansion in *Heliconius* [129,130]. Indeed, recent work shows expansion of the mushroom bodies is primarily driven by increases in the region of the calyx receiving visual, rather than olfactory, input (Couto et al, *in prep.*). This is consistent with *Heliconius* relying heavily on visual learning and memory. However, direct evidence for a functional link between the mushroom bodies and visually-oriented spatial memory is limited to a handful of
ablation experiments in cockroaches [97] and ants [98,99]. Indirect evidence also comes from comparative data from Hymenoptera, where expansion of the mushroom bodies coincided with the evolution of parasitoidism [21], a strategy that relies on spatial memory for host location [208]. Plasticity experiments in a desert ant also showed visually-guided foraging experience affecting mushroom body maturation [209]. Though suggestive, these data are relatively impoverished compared to our understanding of the role of the central complex, another sensory-motor integration structure in the central brain, in insect spatial learning and orientation [210,211].

Comparatively testing the spatial learning abilities of *Heliconius* and outgroup Heliconiini could provide insight into the role of the mushroom body in spatial learning. However, there is only very limited data on the relative cognitive abilities of *Heliconius* non-pollen-feeding Heliconiini [277]. I therefore aimed to compare the performance of *Heliconius melpomene* and *Dryas iulia* in learning the location of a food reward in a large-scale T-maze. I hypothesised that the expanded mushroom bodies of *Heliconius melpomene* would be associated with superior spatial learning. Mazes have been used to test spatial learning abilities across a wide range of taxa, with maze learning having been described in vertebrates [282–284], cephalopods [285,286] and several arthropods including bees [287], ants [288] and decapods [289,290]. However, to my knowledge, the present study is the first to experimentally test spatial learning and memory in any Lepidoptera.

### 3.2 Methods

1. **Pupal supply and T-maze environment**

*Dryas iulia* and *Heliconius melpomene* pupae were shipped to Moulis, France from the Stratford Butterfly Farm (UK) and the Costa Rica Entomological Supply. Pupae were then incubated at 30°C and 80% humidity until emergence.

Behavioural experiments were performed between June and August 2019, when conditions are historically warmer, stable, and dry, using the Metatron facility in Ariège, France [291]. The Metatron consists of an open-air grid of 48 10 m x 10 m x 2 m cages, which can be connected by corridors 19 m long (Figure 3.1). The scale of the Metatron allowed me to test the spatial learning abilities of butterflies in a relatively large experimental environment that approaches the spatial scale of *Heliconius* foraging behaviour in the wild [143,197,203,292].
T-mazes were created by connecting three cages in a row and connecting a fourth to the central cage (Figure 3.1). Prior to beginning the experiments, the vegetation in the cages was stripped down to a short grass to remove any nectar or pollen sources. A 1.8 m-tall black cylinder (500 mm diameter) was placed next to the entrance of each corridor as a visual landmark to aid butterflies in identifying corridor entrances (Figure 3.1). The cage mesh is also largely transparent, presumably enabling the butterflies to see the surrounding landscape. A red ribbon was strung 40 cm above the ground through each corridor as a visual attractor to encourage movement between cages. The open-air nature of the Metatron makes it difficult to control the climate within the cages. However, shades were programmed to close when temperatures exceeded 30°C and sprinklers were scheduled to run between 6 am and 7 am each morning to increase humidity. With the base of the T-maze facing to the southwest, the sun would rise over the right arm and set over the left.

**ii) Butterfly maze training and testing**

I aimed to train butterflies to associate a food reward with either the left or right arm of the T-maze. Butterflies were trained in several successive groups, each for four days. For each group, a sugar-pollen (20% sugar, 5% bee pollen, 75% water, w/v) reward, presented in red artificial flowers, was placed in either the right or left arm of the T-maze (Figure 3.1). The reward was placed conspicuously in the centre of the cage but was only visible once butterflies entered the correct cage due to the angle in the corridors connecting each cage. The position of the food reward remained constant throughout the training period. This reward was the only source of food within the T-maze.

Training began by releasing butterflies in the centre of the base cage of the T-maze (Figure 3.1) at 09:30 the morning after emergence. During training, butterflies were free to explore the T-maze for the entire day and could freely feed on the food reward once it was located. As the cages are home to many predatory insects and arachnids, I collected the butterflies each evening after sunset and kept them in a pop-up cage in a room overnight at 27°C to minimise nocturnal predation. During training, each morning I released the butterflies at 9.30 am in the centre of the base cage (Figure 3.1) to ensure that each butterfly began its day of foraging from the same location.

Upon completion of the training period, butterflies were tested individually over the two following days, with the feeders having been removed from the T-maze. During testing, an individual was released in the centre of the base cage and allowed to freely fly through the
T-maze. If the individual flew into either the left or right arm of the T-maze, that choice was recorded and the trial ended. If the butterfly was inactive or had not moved from the base cage after 30 minutes, the trial was stopped. Butterflies were tested sequentially between 09:30 and 14:30, when the sun was high. At the end of each day’s testing period, the food reward was returned to the T-maze and butterflies were released as a group in the base cage to forage for the remainder of the afternoon.

**iii) Statistical analysis**

I tested whether the placement of food in the right or left arm of the T-maze had a significant effect on individual choice to fly left or right. Choice data were analysed in R v 4.1.0 using a generalised linear mixed model (GLMM) using the `glmer` function from the R package `lme4` v 1.1-27.1 [293]. The GLMM was fitted with a binomial distribution, treating the trained food location as a fixed effect and including individual as a random effect. The model was assessed for compliance with statistical assumptions using the R package `DHARMa` [294].

![Figure 3.1](image_url)  
**Figure 3.1** T-maze used in behavioural experiments doe at the Metatron in Ariège, France. Butterflies were always released from the same point during training and testing. Food is shown as presented for butterflies trained to the right arm of the maze. Cages and corridors are drawn to scale.
3.3 Results

i) High levels of mortality

Throughout this experiment, mortality was high for both pupae and adults. Several shipments of pupae were delayed during transit, resulting in very low emergence rates. Consequently, only 14 *Dryas iulia* adults began training. Survival rates for adults were also low, likely due to the effects of transportation as pupae, the open-air nature of the cages rendering butterflies vulnerable to predation and the weather conditions, which were highly variable during the experimental period. None of the adult *Dryas iulia* survived to complete the training, and therefore could not be tested. For *Heliconius melpomene*, adult survival was still low, but 14 individuals out of a total of 38 which started the training, were able to complete the experiment, consisting of three groups of two, seven, and five individuals, trained on right, left, and right, respectively.

ii) *Heliconius melpomene* solve a T-maze

In total, 32 trials were completed by the 14 *Heliconius melpomene*. Due to variation in activity levels between individuals, the amount of completed trials per individual ranged from 1 to 6 (Table 3.1). Individuals trained with food on the right were more likely to fly to the right arm of the maze during testing and vice versa, indicating butterflies were able to learn the position of the food reward (Table 3.1, Figure 3.2, \(\chi^2=6.242\), d.f.=1, \(P=0.0125\)).
Table 3.1 Test results for *Heliconius melpomene* individuals after training in the T-maze. Food placement indicates the arm of the maze in which the reward was placed during training. Right and Left indicate the number of times that direction was chosen first during a flight from the base of the T-maze.

<table>
<thead>
<tr>
<th>ID</th>
<th>Food placement</th>
<th>Flight direction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Right</td>
</tr>
<tr>
<td>54</td>
<td>Left</td>
<td>1</td>
</tr>
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<td>1</td>
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</table>

Figure 3.2 Spatial learning of *Heliconius melpomene* in a T-maze. Total number of *Heliconius melpomene* individuals with a mean preference of left, right or neutral after four-days training in the T-maze. Results are separated by treatment group (left-trained and right-trained). Individuals trained with food on the right were significantly more likely to fly right ($\chi^2=6.2417$, d.f.=1, $P=0.0125$). * = $P<0.05$

3.4 Discussion

I tested whether *Heliconius melpomene* were able to solve a T-maze by training individuals to associate a food reward with either the left or right arm of the maze. The spatial position of the food reward during training did indeed have a significant effect on individuals’ tendencies to
fly left or right during testing, suggesting they were able to learn spatial information about the food reward (Figure 3.2). It was hypothesised that because of their expanded mushroom bodies and traplining behaviour, *Heliconius melpomene* would exhibit superior spatial learning abilities compared to *Dryas iulia*. However, due to the high mortality suffered by both species, but particularly *Dryas iulia*, I was unable to make this comparison.

Nevertheless, these results provide the first evidence of maze-learning in Lepidoptera, adding to a growing body of evidence of maze-solving abilities in arthropods, including in bees [287], ants [288] and decapods [289,290]. The present experiment was conducted using the simplest possible maze, a T-maze involving only one decision point. However, the honeybee *Apis mellifera* [287], the ant *Cataglyphis niger* [288] and the crab *Carcinus maenas* [290], have each been shown to be capable of solving more complex mazes with several decision points. Future experiments could build upon these results by testing *Heliconius melpomene*’s spatial learning abilities in more complex arenas.

These results contrast with a previous study which found no evidence of *Heliconius* spatial learning in a much smaller arena (a 61 x 61 cm grid) [280]. This suggests *Heliconius* may only learn spatial information at larger scales, consistent with the relatively long ranges over which they collect pollen (~1 km²). It is also an important reminder of the need for conducting ecologically-relevant experiments when investigating cognition [41]. This difference in spatial learning ability at different scales also raises questions about *Heliconius*’ mechanisms of navigation.

In insects, tools of navigation include sun and magnetic compasses, path integration and visual landmarks [295–297]. Although these results indicate *Heliconius melpomene* were able to learn the location of the food reward in the T-maze, the mechanisms underpinning their navigation of the maze are unclear. A recent study showed experimentally displaced *Heliconius* will return to their original locations, quickly orienting themselves in the correct direction, although their mechanism of navigation also remains similarly unknown [205]. Among Lepidoptera, the use of a time-calibrated sun compass is known in Monarch butterflies [296,298]. During this experiment, the sun was clearly visible through the cage mesh, and may have been used as a navigational aid. Cages were oriented with the base cage facing southwest, so the sun moved in a general right to left direction over the maze throughout the day. As butterflies were tested in a five-hour window, the position of the sun inevitably changed between individual trials, although the sun was generally high in the sky during testing.
Alternatively, path integration involves an animal monitoring the directions and distances it travels, thereby estimating their position relative to a starting location [299]. This strategy is used by a diverse range of insects including honeybees, flies, cockroaches and ants [299], but has not been shown in Lepidoptera. Lastly, *Heliconius melpomene* may have been using visual landmarks to navigate the maze, a method of navigation well-studied in Hymenoptera [98,208,300–303]. I aimed to minimise reliance on visual landmarks, by placing identical black cylinders at each corridor entrance so that no unique landmark within the testing arena would be associated with the food reward. However, since the cages consisted of semi-transparent mesh, the potential use of landmarks external to the cage cannot be precluded. Further experimentation is necessary to determine the mechanisms by which *Heliconius melpomene* navigate and learn spatial information.

In conclusion, the present results show that *Heliconius melpomene* can learn the location of a food reward in a T-maze and is the first demonstration of maze-learning in Lepidoptera. Due to high mortality, however, the spatial learning abilities of *Heliconius melpomene* could not be compared with *Dryas iulia*. Further comparative studies across *Heliconius* and non-pollen-feeding Heliconiini are necessary for investigating a potential association between the expansion of the mushroom body and enhanced spatial memory. Such studies could also benefit from testing butterflies in mazes of varying complexity. In addition, further experiments are necessary to identify the mechanisms by which *Heliconius melpomene* were able to navigate the maze.

The high butterfly mortality during the experiment placed an unfortunate limitation on the amount of data that could be collected during the limited time within which I had access to the Metatron facility. These high mortality rates were primarily due to extremely variable weather conditions, the continued presence of predators in the cages despite attempts to remove them, and a reliance on store-bought pupae, which are generally less robust due to variable conditions during shipping. These issues could largely be avoided by conducting a similar scale experiment in a tropical environment. However, no suitable facility currently exists. An alternative approach is to assess specific behaviours that could be important for spatial learning in *Heliconius* such as long-term memory, reversal learning and elemental learning. This allows for a reduction in the complexity of the experiments, making comparative assays across species more tractable. I pursue this approach in Chapters 5 and 7 which present comparative experiments between *Heliconius* and non-*Heliconius* Heliconiini testing discrete cognitive abilities.
4 No evidence for a role of host plant use in mushroom body expansion in Heliconiini butterflies

4.1 Introduction

Herbivorous insects, which generally specialise on a limited range of plants, must solve the ecological challenge of identifying suitable host plants in complex, heterogenous environments [304]. It has been proposed that some visually-oriented insects may identify host plants based on either a learned or innate “search image” for particular leaf shapes [305,306]. Evidence for shape recognition in insects has primarily come from studies on foraging bees [307–310]. However, within butterflies, Eurema brigitta exhibit a preference for leaves shaped like those of their host [311], while shape learning has been demonstrated in Danaus plexippus in a feeding context [218], and in Battus philenor and Heliconius erato in oviposition contexts [214,219,306,312].

The expansion of the mushroom bodies (MBs) in Heliconius has thus been speculatively linked with the cognitive demands of identifying and learning host plants [213]. Heliconiini butterflies lay almost exclusively on Passiflora host plants, except for the “Neruda” clade of Heliconius which exploit Dilkea species [213]. Passiflora display a remarkable diversity of leaf shape [313], and host plant choice in H. erato has been shown to be partly based on leaf-shape recognition and learning, with shape preferences able to be associatively conditioned when presented with olfactory cues [214]. It has even been suggested that the leaf shape diversity of Passiflora was driven by the visual identification of host plants by Heliconius [213,214]. Leaf shape, in addition to olfactory cues, therefore, seems to play a role in the locating of suitable host plants by Heliconius butterflies. Given that many Heliconius species can lay on a range of Passiflora species, there may have been selection for the ability to efficiently learn and remember leaf-shape search images for the suitable host plants species that are locally common. It is, therefore, possible that the expanded mushroom bodies of Heliconius was driven by selection for an enhanced shape learning ability to facilitate improved
identification of appropriate host plants. If this were the case, *Heliconius* could be expected to exhibit superior shape-learning ability relative to non-*Heliconius* Heliconiini. However, shape learning has only been assessed in a single *Heliconius* species, *H. erato* [214].

The MBs are known to play a role in visual learning in several groups of insects, including honeybees, ants and cockroaches [90,97–99], but have not been directly linked to shape-learning ability. However, within butterflies, there is some limited evidence linking the mushroom bodies to host plant use and learning. When exposed to host plant environments of varying complexities, the relative generalist butterfly *Polygonia c-album* exhibited greater MB plasticity than two relative specialists, *Aglais io* and *Aglais urticae* [215]. Similarly, in the cabbage white butterfly, *Pieris rapae*, larger mushroom body calyces were associated with an improved ability to locate difficult-to-learn red host plants, and experience with red hosts was positively related to increased mushroom body lobe size [216]. In Heliconiini, the degree of host plant specialisation varies markedly [314], and it is possible that variation in relative MB size across the clade may be correlated with the phenotypic diversity of host plants exploited.

Here, I test whether MB expansion in *Heliconius* is related to shape identification and learning using two complementary methods:

i. comparative shape learning experiments across six Heliconiini species using artificial feeders, and;

ii. phylogenetic comparative analyses testing whether variation in relative MB size across the Heliconiini is associated with the species and morphological diversity of host plants exploited.

Firstly, I test whether individuals from three non-*Heliconius* species, *Dryas iulia*, *Dryadula phaetusa* and *Agraulis vanillae*, and three *Heliconius* species, *H. hecale*, *H. melpomene*, and *H. erato* can learn to associate a food reward with an unattractive “diamond” shape, over their preferred “star” shape [214] (Figure 4.1). *H. erato* has been shown to learn shape information from both floral and host plant stimuli [214], and artificial feeders provide a tractable experimental system. I hypothesise that, if MB expansion is linked to shape learning, *Heliconius* species, generally, would outperform the non-*Heliconius* species in this task. Secondly, I assessed whether relative MB size across the Heliconiini correlates with the number of host plants used in a phylogenetic comparative framework. However, given that many *Passiflora* species exhibit similar leaf shapes, host plant number alone has limitations as a proxy for the diversity of leaf shapes exploited. Therefore, for each Heliconiini species, I also
quantified the morphospace of host plant leaf shapes using Elliptical Fourier Descriptors (EFDs) [313]. I then tested whether differences in host plant morphospace explain any variation in relative MB size across the Heliconiini. Again, if diversity of host plant use drove MB expansion, I might expect to find a positive association with leaf shape diversity if generalisation is cognitively demanding, or potentially a negative association if specialisation on host plant search images is a more complex task.

4.2 Methods

i) Animal husbandry

Associative shape learning experiments were carried out on captive-reared butterflies between January and April 2018 at the STRI Insectaries in Gamboa, Panama. All individuals used in the shape-learning experiments were freshly eclosed and reared from stocks established with locally-caught, wild butterflies using the insectaries at the Smithsonian Tropical Research Institute (STRI) in Gamboa, Panama. Stock butterflies were kept in 2x2x3 m mesh cages in ambient conditions with natural light. Larvae were reared in mesh pop-ups and were provided with fresh leaves daily. *H. erato, Dryas iulia, Dryadula phaetusa* and *Agraulis vanillae* were reared on *P. biflora, H. melpomene* on *P. triloba* and *H. hecale* on *P. vitifolia*. Training and testing of butterflies were conducted in 2x2x3 m mesh cages in ambient conditions under natural light. A single *Psychotria elata*, with all flowers removed, was placed in the rear right corner of these cages as a roosting site.

ii) Shape-learning experimental protocol

The day after eclosion, butterflies were transferred to a pre-training cage to familiarise them with the use of artificial feeders. Here, individuals were fed solely with red, circular feeders (Figure 4.1(a)), filled with a sugar-protein solution (20% sugar, 5% Vertark Critical Care Formula, 75% water, w/v) for one day. Artificial feeders were made from coloured foam with a centrally placed 0.5 ml Eppendorf tube. Throughout the experiment, all feeders were presented with a large, circular, green background so that the silhouette of the red shapes was clearly delineated in a consistent manner.
After pre-training, butterflies were introduced to a testing cage to determine initial preference between two shapes – a “diamond” and a “star” shape (Figure 4.1). The choice of these shapes was based on a previous study showing that *H. erato* can distinguish between them and tend to prefer star-shaped feeders over the diamond shape [214]. The testing cage contained 3 feeders of each shape, arranged randomly for each trial, and separated from each other by 15 cm (Figure 4.2). To ensure that butterflies responded to visual cues only, feeders in the testing cages were empty. Preference testing lasted for four hours from 08:00 to 12:00 and was filmed using a GoPro Hero 5 camera mounted to a tripod. The film was then reviewed to count the number of feeding attempts per individual on each shape, with up to 40 attempts recorded. A feeding attempt was only counted if the butterfly landed on the feeder and probed it with its proboscis.
Figure 4.2 Example of feeder arrangement during preference trials, showing the two different shapes used. Feeders were evenly spaced, but randomly arranged for each trial.

Butterflies were then placed in a training cage for ten days which contained diamond-shaped feeders filled with sugar-protein solution and star-shaped feeders filled with an aversive quinine solution (Figure 4.3). Through this combination of positive and negative stimuli I aimed to condition the butterflies to favour the diamond-shaped feeders over the star shapes. This training period lasted for 10 full days, after which the trained feeding preferences were tested following the same protocol as the initial preference test.
Figure 4.3 Example of feeders in a training cage showing a Heliconius hecale individual feeding. Equal numbers of each shape were placed randomly around the cage and butterflies were free to feed ad libitum. Butterflies were marked with an ID tag on each hindwing.

iii) Shape learning statistical analyses

Shape preferences and learning performance were analysed with generalised linear mixed models (GLMMs) using a binomial distribution with the *glmer* function from the package *lme4* v 1.1-27.1 in R v 4.1.2 [293]. Diagnostics for these GLMMs were assessed using the R package *DHARMa* v 0.4.4 [294]. All post hoc comparisons were made by obtaining the estimated marginal means using the R package *emmeans* v1.7.0 and were corrected for selected multiple comparisons using the Tukey test [315]. Interspecific differences in initial shape preference were tested using a GLMM with species as a fixed effect and an individual-level random effect. For each species, I then tested whether initial preference towards a certain shape differed significantly from random using a GLMM with only individual-level random effects. Interspecific differences in shape learning performance were tested for using a GLMM with species and training as fixed effects, with an individual level-random effect. I also tested for an overall difference between Heliconius and non-Heliconius individuals using a GLMM with membership in Heliconius and training as fixed effects, with individual and species-level random effects. Sex was also initially included as a fixed effect in these models, but was non-significant and so removed.
iv) Host plant number and quantification of leaf morphospace

Using the neuroanatomical dataset described in Chapter 2, combined with Heliconiini host plant data collated in Kozak (2015) [314], I investigated whether the volumes of the mushroom body (MB), antennal lobe (AL) and medulla (ME) varied with host plant use, controlling for the size of the rest of the central brain (rCBR). In total, there were 39 Heliconiini species for which I had both host plant and neuroanatomical data. I ran a series of phylogenetic GLMMs with the R package *MCMCglmm* v 2.32 [254], testing whether the volumes of these brain regions varied with the number of host plants used. These analyses used the unpublished Heliconiini tree from Chapter 2, generated by Francesco Cicconardi.

However, simply taking the number of host plants exploited is an imperfect characterisation of the complexity of a butterfly species’ visual interactions with host plants, given several host plant species could present a similar search image. Accordingly, I also used morphometric analysis to quantify the morphospace of host plant leaf shapes used by Heliconiini species, following Chitwood & Otoni (2017) [313]. I used the program *SHAPE* v 1.3 [316,317] to characterise the outlines of individual host plant leaves with (Elliptical Fourier Descriptors) EFDs, which have previously been used to describe leaf shape [318–321], including in *Passiflora* [313,322]. Leaf images were sourced from the Global Plants database managed by JSTOR, the Encyclopedia of Life and the digital collections of the Muséum national d'histoire naturelle, Paris, and the Royal Botanic Gardens, Kew. These images were cropped to images of single leaves and edited to remove the petiole and non-leaf material (Figure 4.4). In total, I collected images for 686 leaves from 150 *Passiflora* species and 19 leaves from 11 *Dilkea* species.

Images were processed in SHAPE which binarizes the images and performs a chain-code analysis (Figure 4.4) [317]. The chain-code file was then used to calculate normalised EFDs based on 40 harmonics, while manually ensuring the bases of each leaf outline were consistently aligned. The R package *Momocs* v 1.3.2 was used to convert the resulting normalised EFD .nef file into a COE object, which was then subject to principal component analysis using *Momocs* [323]. Using Heliconiini host plant data collated in Kozak (2015) [314], with the R package *dispRity* v 1.6.0 I calculated the four-dimensional volume of host plant morphospace exploited for each Heliconiini species, based on the first four principal components. The four-dimensional morphospace volumes were then square-root transformed for all further analyses to better fit a normal distribution. I then conducted a series of
phylogenetic GLMMs with *MCMCglmm* to test for relationships between host plant morphospace and the relative volumes of brain regions [254]. Controlling for rCBR volume, I tested whether MB, AL or ME volume varied with either the number of host plants used, or four-dimensional host plant morphospace, controlling for allometric effects using rCBR, while also testing whether these relationships varied between *Heliconius* and non-*Heliconius* species. Finally, host plant use can vary between populations within a Heliconiini species. Therefore, I further tested for these relationships within a specific Heliconiini community (14 species) with known host plant use in Gamboa, Panama, and the nearby Soberanía National Park [324]. All *MCMCglmm* models described above were run for 500,000 iterations, with a burn-in of 10,000 and a thinning factor of 500.

![Image processing pipeline for leaf shape analysis](image)

**Figure 4.41** Image processing pipeline for leaf shape analysis in SHAPE. Firstly, the cropped leaf image is edited to remove the petiole and non-leaf material, the image is then binarized and lastly chain code analysis of the shape outline is performed. Example shows a *Passiflora capsularis* leaf.

### 4.3 Results

**i) No evidence *Heliconius* are better shape learners than other Heliconiini**

All six species showed a significant initial preference for the star-shape feeders over the diamond feeders, with stars accounting for approximately two-thirds of all feeding attempts (Table 4.1, Figure 4.5). Initial shape preference did not significantly differ between species ($\chi^2=2.709$, d.f.= 5, $P=0.746$).
Table 4.1 Outputs for each species from generalised linear mixed models testing initial individual preferences against a null hypothesis, with an individual-level random effect. All species significantly preferred star-shaped feeders over diamonds.

| Species              | z value | Pr(>|z|)   | Preferred shape |
|----------------------|---------|-----------|-----------------|
| Dryas iulia          | -9.368  | <0.0001***| Star            |
| Dryadula phaetusa    | -6.497  | <0.0001***| Star            |
| Agraulis vanillae    | -10.04  | <0.0001***| Star            |
| H. hecale            | -7.045  | <0.0001***| Star            |
| H. melpomene         | -5.912  | <0.0001***| Star            |
| H. erato             | -8.912  | <0.0001***| Star            |

Learning ability, however, varied significantly between species (Tables 4.2 & 4.3, Figure 4.5), but Heliconius as a whole were not superior shape learners than the other Heliconiini (Tables 4.3 & 4.4, Figure 4.5). Rather, shape learning ability was scattered across the phylogeny. Agraulis vanillae, Dryadula phaetusa, H. melpomene and H. erato showed a significant shift in preference towards the diamond shape after training, while Dryas iulia, and H. hecale showed no shift (Table 4.3, Figure 4.5). Notably, even the species that did show a significant shape-learning effect only showed a slight shift in preference and still maintained a bias towards stars over diamonds (Figure 4.5). This low effect size suggests that increasing the difficulty of the shape-learning task would not reveal a difference in shape-learning ability between Heliconius and the outgroup Heliconiini.

Table 4.2 ANOVA test on a generalised linear mixed model with training and species treated, and their interaction, as fixed effects, and an individual-level random effect.

<table>
<thead>
<tr>
<th></th>
<th>χ²</th>
<th>Df</th>
<th>Pr (&gt;χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training</td>
<td>52.174</td>
<td>1</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Species</td>
<td>7.9132</td>
<td>5</td>
<td>0.161</td>
</tr>
<tr>
<td>Training:Species</td>
<td>18.565</td>
<td>5</td>
<td>0.00232**</td>
</tr>
</tbody>
</table>

Table 4.3 Pairwise comparisons between untrained and trained shape preferences for each species based on the generalised linear mixed model presented in Table 4.2, corrected for multiple comparisons using Tukey’s test.

<table>
<thead>
<tr>
<th>Species</th>
<th>z ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dryas iulia</td>
<td>-0.629</td>
<td>0.989</td>
</tr>
<tr>
<td>Dryadula phaetusa</td>
<td>-2.883</td>
<td>0.023*</td>
</tr>
<tr>
<td>Agraulis vanillae</td>
<td>-4.717</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>H. hecale</td>
<td>-0.869</td>
<td>0.946</td>
</tr>
<tr>
<td>H. melpomene</td>
<td>-3.822</td>
<td>0.0008**</td>
</tr>
<tr>
<td>H. erato</td>
<td>-4.949</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>
Figure 4.5 Shape learning in Heliconiini. Boxplots show frequency of feeding attempts on diamond shapes, when given a choice between diamond and star shapes, before and after training for six Heliconiini species. Asterisks indicate species that exhibited a significant change in feeding preferences after 10 days’ training. Shape learning performance varied significantly between species, but *Heliconius* were not superior shape learners overall.
Table 4.4 ANOVA test on a generalised linear mixed model with training and membership of the *Heliconius* genus, and their interaction, treated as fixed effects, in addition to species, individual and observation-level random effects. Training was associated with a shift in shape preference, but *Heliconius* were not better shape learners than outgroup Heliconiini.

<table>
<thead>
<tr>
<th></th>
<th>$\chi^2$</th>
<th>Df</th>
<th>Pr (&gt;(\chi^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training</td>
<td>29.107</td>
<td>1</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>Heliconius</em></td>
<td>0.0616</td>
<td>1</td>
<td>0.804</td>
</tr>
<tr>
<td>Training:<em>Heliconius</em></td>
<td>0.1283</td>
<td>1</td>
<td>0.720</td>
</tr>
</tbody>
</table>

**ii) Diversity of host plant use not associated with mushroom body size in Heliconiini**

There was no relationship between the number of host plants used and mushroom body (MB) size, controlling for the size of the rest of the central brain (rCBR), within the Heliconiini (Table 4.5). This was also true for both antennal lobe (AL) and medulla (ME) volumes (Table 4.5). For MB size, the interaction between number of host plants and membership in the *Heliconius* genus approached significance (Table 4.5). This seems to have been driven by an apparent negative trend, contrary to expectations, between relative MB size and host plant number in the outgroup species (Figure 4.6(a)). However, when the outgroups were reanalysed alone, the relationship between MB size and number of host plants was not significant (pMCMC = 0.234).

Table 4.5 ANOVA test on a series of phylogenetic generalised linear mixed models analysing the relationships between host plant number and mushroom body, medulla, and antennal lobe volumes, controlling for allometric effects using the volume of the rest of the central brain (rCBR), across Heliconiini species (n=34). Host plant number was not significantly associated with any of these brain regions.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Factor</th>
<th>pMCMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mushroom body</td>
<td>rCBR</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Number of host plants</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Heliconius</em></td>
<td>0.1263</td>
</tr>
<tr>
<td></td>
<td>Number of host plants:*Heliconius</td>
<td>0.0822</td>
</tr>
<tr>
<td>Medulla</td>
<td>rCBR</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Number of host plants</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Heliconius</em></td>
<td>0.952</td>
</tr>
<tr>
<td></td>
<td>Number of host plants:*Heliconius</td>
<td>0.593</td>
</tr>
<tr>
<td>Antennal lobe</td>
<td>rCBR</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Number of host plants</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Heliconius</em></td>
<td>0.0822</td>
</tr>
<tr>
<td></td>
<td>Number of host plants:*Heliconius</td>
<td>0.375</td>
</tr>
</tbody>
</table>
Figure 4.6 Relative mushroom body (MB) size in Heliconiini species (n=34) plotted against (a) number of host plant species and (b) leaf shape diversity of those species. Residual MB size was calculated with a phylogenetic regression of MB size against the size of the rest of the central brain (rCBR) using the phyl.resid function in the R package phytools. There is no significant relationship between relative MB size and number of host plants for either Heliconius or the outgroups.

I attempted to better characterise the complexity of Heliconiini visual interactions with host plants by estimating the morphospace of host plant leaf shapes exploited by a species. Principal component analysis of normalised EFDs based on 40 harmonics revealed that 93.3% of variation in Passiflora and Dilkea spp. host plant leaf shape is explained by the first four principal components, accounting for 60.8%, 17.8, 11.1% and 3.6% of variation, respectively (Figures 4.7 & 4.8). There was a significant positive relationship between the number of host plants used by a species and the leaf-shape morphospace covered by those plants (t=11.445, d.f.=32, P<0.0001, Figure 4.9). The $R^2$ was 0.798, with several marked outliers, demonstrating the value in accounting for phenotypic diversity of host plants, rather than simply their number, in characterising a species’ search image landscape (Figures 4.9 & 4.10). However, as with host plant number, neither MB, AL, or ME volumes showed any relationship with host plant morphospace (Figure 4.6(b), Table 4.6). This was true for both Heliconius and non-Heliconius Heliconiini. When this analysis was applied to a specific Heliconiini community (14 species) around Gamboa, Panama, and the Soberanía National Park [324]. Again, neither host plant
number (pMCMC=0.645) or leaf shape diversity (pMCMC=0.878) was significantly associated with relative mushroom body size.

Figure 4.7 Shape variation in Heliconiini host plants. Principal components (PCs) representing shape variance in 150 *Passiflora* and 10 *Dilkea* species based on Elliptical Fourier Descriptor analysis. Eigenleaf representations at ±1 and ±2.5 SD are shown for the first four PCs. Percent variance explained by each PC indicated.

Figure 4.8 Principal component analysis of leaf shape variation in 150 *Passiflora* and 11 *Dilkea* Heliconiini host plants, characterised by Elliptical Fourier Descriptors. (a) PC1 vs PC2. (b) PC3 vs PC4. Each point represents an individual leaf.
Figure 4.9 Relationship between number of host plant species used and leaf shape morphospace of those plants across Heliconini species. Dashed line shows linear regression ($t=11.445$, d.f.=32, $P<0.0001$, $R^2=0.798$).

Figure 4.10 Example of the difference between host plant number and disparity of host plant leaf shape. *Heliconius hecale* uses fewer host plants than *H. ethilla*, but the leaf shape morphospace of those plants is far larger. Each point represents a single leaf sample.
### Table 4.6

ANOVA test on a series of phylogenetic generalised linear mixed models analysing the relationships between host plant leaf shape morphospace and mushroom body, medulla, and antennal lobe volumes, across Heliconiini species (n=34), controlling for allometric effects using the volume of the rest of the central brain (rCBR).

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Factor</th>
<th>pMCMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rCBR</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Mushroom body</td>
<td>Host plant morphospace</td>
<td>0.200</td>
</tr>
<tr>
<td></td>
<td>Heliconius</td>
<td>0.00601*</td>
</tr>
<tr>
<td></td>
<td>Host plant morphospace:Heliconius</td>
<td>0.317</td>
</tr>
<tr>
<td>Medulla</td>
<td>rCBR</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Host plant morphospace</td>
<td>0.685</td>
</tr>
<tr>
<td></td>
<td>Heliconius</td>
<td>0.984</td>
</tr>
<tr>
<td></td>
<td>Host plant morphospace:Heliconius</td>
<td>0.683</td>
</tr>
<tr>
<td>Antennal lobe</td>
<td>rCBR</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td></td>
<td>Host plant morphospace</td>
<td>0.0982</td>
</tr>
<tr>
<td></td>
<td>Heliconius</td>
<td>0.0681</td>
</tr>
<tr>
<td></td>
<td>Host plant morphospace:Heliconius</td>
<td>0.435</td>
</tr>
</tbody>
</table>

### 4.4 Discussion

The six Heliconiini species assessed in this study exhibited a clear ability to distinguish between shapes, all showing a marked preference for star-shaped feeders over diamonds, consistent with previous experiments showing a similar bias in *H. erato* (Figure 4.5) [214]. This preference is likely explained by the resemblance of the star-shape to the radial symmetry of the flowers typically exploited by these Heliconiini [133,138,139]. Visual cues, including “search images”, therefore appear to play an important role for Heliconiini in identifying key resources in their environment. Notably, naïve Heliconiini possess an inherent attraction towards certain shapes, similar to previous findings in honeybees [310] and the butterflies *Battus philenor* and *Eurema brigitta* [306,311].

These results corroborate previous findings of shape learning in *H. erato* [214], while also demonstrating comparable abilities in *H. melpomene* and the non-*Heliconius* Heliconiini *Agraulis vanillae* and *Dryadula phaetusa* (Figure 4.5). There was significant variation in shape-learning performance between species, as neither *H. hecale* and or *Dryas iulia* showed any evidence of having learned the shape cue. Contrary to expectations, *Heliconius*, as a group, were not superior shape learners than the non-*Heliconius* species (Table 4.4). The lack of difference in shape-learning performance between *Heliconius* and non-*Heliconius* Heliconiini, suggests that mushroom body (MB) expansion in *Heliconius* is not associated with enhanced
shape learning ability. This result, thus, casts doubt over the hypothesis that the expanded mushroom bodies of *Heliconius* are adapted for shape learning related to the visual recognition of *Passiflora* host plants.

In addition, even amongst the Heliconiini species which did exhibit a significant shape learning effect, the frequency of feeding attempts on diamond shapes remained below 50% (Figure 4.3). The inherent preference for the star shape appears very difficult to alter through associative conditioning, even when the star shape is presented with an aversive stimulus. This suggests that visual identification of resources in Heliconiini butterflies may be driven more by innate search images than learned shapes. A similarly fixed preference was seen in the selection of host plants by *H. erato phyllis*, which was not able to be altered by experimental conditioning [325]. The present results can also be contrasted with the apparent ease with which Heliconiini can learn, and modify, colour associations [217]. In other insects, it has similarly been observed that the monarch butterfly [218] and honeybees [326,327] also learn colour much more readily than shape. Conversely, the Hymenopteran parasitoid *Microplitis croceipes* learns shape more readily than colour [328]. This variation in the ease with which species learn shape cue provides reminder that cognition evolves in response to particular ecological challenges [4,41].

One caveat of this experiment is that to test shape learning in an interspecific, comparative framework, I conducted the experiment in a feeding, rather than oviposition context. The Heliconiini involved in this study use of a range of different host plants with varying leaf shapes and using the same shape cues for each butterfly species would not have been possible in an oviposition context. Additionally, testing the learning abilities of butterflies in a feeding context also has the advantage of far greater tractability, permitting the sample sizes necessary for a comparative behavioural experiment across six species. Although in some cases the importance of different types of cues can be context dependent [120,329,330], the shape learning abilities of *H. erato* translate across both feeding and oviposition contexts [214]. On this basis, I suggest that shape-learning performance in a feeding context should be interpreted as a measure of general shape-learning ability in Heliconiini butterflies. However, combining the shape-learning experiment with comparative analyses of host plant diversity provides a complementary, alternative test of the overriding hypothesis.

Consistent with the results of the shape-learning experiment, phylogenetic comparative analysis showed no evidence of association between MB size and the number or leaf shape
diversity of a species’ host plants. Additionally, neither antennal lobe (AL) or medulla (ME) volumes showed any such relationship. This is somewhat surprising given the role of these neuropils in processing olfactory and visual information, respectively. Worth noting is that host plant use can vary between populations for some Heliconiini species [325], and *Passiflora* species diversity within a given area can be limited to approximately 10 species [133]. Therefore, for some Heliconiini populations, the present study is likely to overestimate host plant number and their leaf shape diversity. Nevertheless, a host plant generalist at the species level is expected to be a generalist at the population level, and likewise with specialists. I further accounted for this potential discrepancy by analysing host plant use within a single Heliconiini community near Gamboa, Panama and the nearby Soberanía National Park [324]. The results of this analysis reflected the results of the wider analysis, also showing no relationship between differences in mushroom body size and host plant number or leaf shape diversity. These results suggest that the identification of host plants based on leaf shape has not been an important factor influencing brain evolution in the Heliconiini, at least at a volumetric scale. Indeed, while shape cues are used by butterflies to detect host plants [214,219,306,311], olfactory cues are likely more important, although these are probably used at shorter distances [304]. Indeed, in a previous study, although *H. erato* trained on a certain host plant leaf shape approached that shape more frequently, the total landings and number of eggs laid did not differ between shapes [214].

Taken together, the shape learning experiment and phylogenetic comparative analyses presented here do not support the hypothesis that visual identification of host plants based on leaf shape has been an important factor in driving MB expansion in *Heliconius*. The other major hypothesis accounting for this elaboration of the MBs is that it was driven by the cognitive demands of trapline foraging for pollen. In Chapters 5 and 7, I present comparative behavioural experiments across the Heliconiini testing cognitive abilities presumably important for traplining – visual long-term memory, reversal learning and non-elemental associative learning.
5 Long-term memory and reversal learning abilities in the Heliconiini

5.1 Introduction

Results from the preceding chapter suggest that the elaboration of the mushroom bodies in *Heliconius* is not associated with host plant use. The primary alternative hypothesis to explain this brain expansion is that it was driven by the cognitive demands of *Heliconius*’ novel foraging strategy – pollen feeding [129,130,134]. Field studies document *Heliconius* butterflies establishing “traplines”, routes along which specific plants are regularly visited, and suggest an advanced capacity for spatial navigation, likely based on learned visual landmarks [133,142,143]. Such behavioural innovations are generally associated with changes in the structure and function of the brain [4]. For example, brain expansion is linked to foraging innovations in several vertebrate groups, including birds and primates [47,73]. In Chapter 3, I demonstrated that *Heliconius* are capable of solving a T-maze over a relatively large travel distance (~60 m) and are capable of learning spatial information. However, conducting comparative studies of Heliconiini spatial learning ability over the larger scales at which wild *Heliconius* foraging (~1 km²) presents several logistical challenges.

Nevertheless, traplining involves several cognitive requirements that are more readily tested in a comparative context across a representative selection of Heliconiini. Firstly, pollen feeding in *Heliconius* appears to depend on an enhanced capacity for long-term memory, as traplines are followed faithfully through time – on the scale of months [143], well past the maximum longevity of non-pollen feeding relatives [140]. *Psiguria* flowers contain large amounts of pollen, and inflorescences generally produce a new flower every 1-3 days [133]. In contrast to the seasonal pollen production common for neotropical angiosperms [200], individual *Psiguria* plants, and even individual inflorescences, can flower continuously for up to a year [133]. A single *Psiguria* plant is therefore potentially a reliable pollen resource for the entire lifespan of an individual butterfly. Despite their value as a pollen resource, *Psiguria* are also relatively scarce, and although predictably rewarding over the long-term, can vary
markedly in flower production from day to day [133]. Therefore, the capacity to maintain stable, long-term memories of pollen resources, even when they become temporarily unavailable, seems to be a prerequisite for efficient traplining for pollen in *Heliconius*.

As centres of learning and memory in the insect brain [83], expanded mushroom bodies could be expected to support enhanced long-term memory. Indeed, the mushroom bodies play a key role in the formation of olfactory long-term memory in *Drosophila*, honeybees and leaf-cutting ants [88,89,96]. The mushroom bodies are also involved in visual learning and memory in several groups of insects, including honeybees, ants and cockroaches [90,97–99]. Yet, the mushroom bodies have not been directly linked to the maintenance of long-term visual memories specifically. More broadly, investment in learning and long-term memory, and the neural tissue that supports it, is costly [54,216,227], but appears favoured by an increase in longevity [55–57], as observed in *Heliconius*. Importantly, mushroom body expansion in *Heliconius* is driven primarily by visual rather than olfactory inputs, which may facilitate enhanced visual long-term memory in particular (Couto et al, in prep.). This was demonstrated by measuring the sizes of the visual and olfactory regions of the mushroom body calyx in *Heliconius* by staining neurons in the ventral lobula of the optic lobe and the antennal lobe and tracing those neurons into the calyx.

Efficient traplining likely also requires the altering of an established route as the environment changes over time (such as when an important resource or landmark is removed) [142]. This would require updating previously learned information, which may depend on advanced reversal learning. In a reversal learning assay animals are trained in an associative learning task. The cue-reward presentations are then later reversed, requiring the animal to inhibit the learned response to the originally rewarded cue and switch to a previously unrewarding, or aversive, cue. This switch is believed to be more cognitively challenging than learning the initial association [331–333]. More generally, reversal learning has also been used as a proxy for cognitive ability in vertebrates [332,334–338], with some studies also finding links between reversal learning ability and brain size [331]. Among insects, reversal learning of visual cues has been demonstrated in honeybees and Monarch butterflies [100,339]. In honeybees, the mushroom bodies appear to mediate the updating of learned information [100] and blocking the function of mushroom bodies impairs reversal learning, but not simple associative learning [101]. I therefore hypothesise that the enlarged mushroom bodies of *Heliconius* may also be associated with an improved reversal learning ability relative to other Heliconiini.
I conducted comparative long-term memory and reversal learning trials across six Heliconiini species – three *Heliconius* (*H. erato, H. melpomene* and *H. hecale*) and three non-*Heliconius* (*Agraulis vanillae, Dryadula phaetusa* and *Dryas iulia*) using coloured artificial flowers associated with either a food reward or an aversive stimulus (quinine). Based on the cognitive demands of trapline foraging, I predicted that the expanded mushroom bodies of *Heliconius* would be associated with improved performance in both the long-term memory and reversal learning tasks.

### 5.2 Methods

Long-term memory (LTM) and reversal learning (RL) experiments, using colour cues, were carried out on captive-reared butterflies between January and April 2019 in Gamboa, Panama. Both experiments used three species of *Heliconius* (*H. erato, H. hecale* and *H. melpomene*) and three species of non-*Heliconius* Heliconiini (*Dryas iulia, Dryadula phaetusa* and *Agraulis vanillae*). The experiments used two colours, purple and yellow, colours chosen based on previous experiments using *H. erato* which showed that neither colour was particularly attractive [217]. The experiments used five-pointed, star-shaped, artificial feeders made from coloured foam, 3 cm in diameter, with a centrally placed 0.5 ml Eppendorf tube that could be filled with liquid (Figure 5.1). Separate individuals were used for the long-term memory and reversal learning trials.

**i) Animal husbandry**

All larvae were reared from stocks established with locally caught, wild butterflies using the insectaries at the Smithsonian Tropical Research Institute in Gamboa, Panama. Stock butterflies were kept in 2x2x3 m mesh cages in ambient conditions with natural light. Larvae were reared in mesh pop-ups and were provided with fresh leaves daily. *H. erato, Dryas iulia, Dryadula phaetusa* and *Agraulis vanillae* were reared on *P. biflora, H. melpomene* on *P. triloba*, and *H. hecale* on *P. vitifolia*. Training and testing of butterflies was conducted in 2x2x3 m mesh cages in ambient conditions under natural light. A single *Psychotria elata*, with all flowers removed, was placed in the rear right corner of these cages as a roosting site.
ii) Pre-training, initial preference testing and initial colour training

For both the long-term memory and reversal learning experiments, individuals were transferred to a pre-training cage one day after eclosion. Here, butterflies were fed solely with white artificial feeders containing a sugar-protein solution (20% sugar, 5% Vertark Critical Care Formula, 75% water, w/v) for two days (from 08:00 to 12:00) to familiarise them with the use of artificial feeders.

After pre-training, butterflies were introduced to a testing cage to determine initial feeding preferences between purple and yellow. Testing cages contained 12 purple and 12 yellow feeders (Figure 5.1) arranged randomly in a 4 X 6 grid, with 6.5 cm between feeders on each side. To ensure that butterflies responded exclusively to visual cues, feeders in the testing cages were empty. Preference testing lasted for four hours from 08:00 to 12:00 and was filmed from above using a GoPro Hero 5 camera mounted on a tripod. Butterflies were individually numbered on their wings for identification using a permanent marker. The film was then reviewed to count the number of feeding attempts per individual on each colour, with up to 40 attempts recorded per individual. A feeding attempt was only counted if the butterfly landed on the feeder and probed it with its proboscis.

![Purple and yellow feeders](image)

*Figure 5.1* Purple and yellow feeders used for the long-term memory and reversal learning assays.

Butterflies were then trained to associate a food reward with their non-favoured colour, based on the results of their initial preference test. For butterflies that initially preferred purple, the training cage contained yellow feeders containing a sugar-protein solution, and purple feeders containing a saturated quinine solution, an aversive stimulus. The opposite arrangement was employed for individuals that initially preferred yellow. This training period lasted for four full days (Figure 5.2). After training, butterfly preferences were re-tested, following the same protocol as the initial preference test, to verify that individuals had indeed acquired the colour-
food association. At this point the long-term memory and reversal learning protocols diverge (Figure 5.2).

![Figure 5.2 Timeline of the long-term memory and reversal learning protocols.](image)

### iii) Long-term memory protocol

After the trained preference test, individuals participating in the long-term memory assay were placed for eight days in a cage identical to the pre-training cage, containing only white feeders filled with a sugar-protein solution. The deprivation of colour stimuli for eight days allowed for testing the long-term memory retention of the colour-food association acquired during the training period, and ensured that long-term memory was being tested rather than short-term or mid-term memory [340]. A period of eight days was chosen because *Heliconius* are known to maintain their foraging routes over periods ranging from weeks to months [132,133,143,292], during which time a pollen resource could be unproductive for several days due to competition or damage, but ultimately rewarding over the long term. Butterflies were then subject to a third preference test to determine if the learned preference was maintained, following the same protocol as the initial preference test (Figure 5.2). *H. melpomene, H. hecale, Dryadula phaetusa* and *Agraulis vanilla* individuals were also subject to an additional extended long-term memory test by placing them in a cage with only white for a further four days before a final preference test.

### iv) Reversal learning protocol

Following the trained preference test, individuals participating in the reversal-learning assay were then trained on the opposite colour to their initial training period. Individuals that had been trained to associate purple with food were placed in a cage containing only yellow feeders filled with sugar-protein and purple feeders filled with quinine, and vice versa for butterflies initially trained on yellow. After a further four days of training, individuals were subject to
another preference test. Butterflies were then trained for another four days under their initial training regime, before a fourth and final preference test (Figure 5.2). A period of four days was chosen for each training period in order to represent the larger time scales over which a pollen resource may become unrewarding for an individual Heliconius [142].

v) Statistical analysis

Long-term memory and reversal learning performance were analysed using generalised linear mixed model (GLMM) analyses using the glmer function from the lme4 package v1.1-21 in R v 4.1.0 [293]. All models used a binomial distribution and treated trial as a fixed effect. When testing for interspecific differences, species was included as a fixed effect. To test for differences between Heliconius and outgroup Heliconiini, membership in the Heliconius genus as a fixed effect with species as a random effect. To test for interspecific differences in the drop in performance between the initial preference test and the long-term memory test, an interaction between species and trial was also included. Diagnostics for these models were assessed using the package DHARMa v0.4.4 [294]. To account for overdispersion, individual and observation-level random effects were also included. Post-hoc comparisons among relevant pairs of species, or tests, were made by obtaining the estimated marginal means using the package emmeans v1.7.0 and were corrected for multiple comparisons using the Tukey test [315]. Significant deviation from random colour preference during the initial preference test and the second long-term memory test was assessed using a null generalised linear mixed model.

For both the long-term memory and reversal learning trials, individuals that exhibited less than 50% accuracy during the initial trained preference test, and therefore did not appear to have learned the food-colour association, were removed from the dataset. In total, 20 individuals scored less than 50% during the first training test and were assumed to have not acquired the trained colour-food associated and removed from the dataset: 1 out of 48 H. erato individuals were removed, in addition to 4 of 52 H. melpomene, 3 of 56 H. hecale, 3 out of 81 Agraulis vanillae, 4 of 63 Dryas iulia, 5 of 78 Dryadula phaetusa. A generalised linear model treating species as a fixed effect did not show significant variation between species in the proportion of individuals scoring less than 50% during the first training test ($\chi^2=2.564$, d.f.=5, $P=0.767$).
5.3 Results

i) Interspecific variation in initial colour preference

Species varied significantly in their naïve colour preferences (Figure 2, $\chi^2=59.59$, d.f.=5, $P<0.0001$). There were no significant differences in the naïve preferences of *H. melpomene*, *H. hecale*, *Dryadula phaetusa* and *Dryas iulia* (Figure 5.3, Table 5.1), which did not significantly differ from 50% (Table 5.2). However, the naïve preferences of *Agraulis vanillae* and *H. erato* significantly differed from the other species and were biased towards purple over yellow (Figure 5.3, Table 5.1, Table 5.2).

![Figure 5.3 Naïve colour preference between purple and yellow for three Heliconius species (H. erato (n=48), H. melpomene (n=52) and H. hecale (n=56)) and three non-Heliconius Heliconiini, Agraulis vanillae (n=81), Dryadula phaetusa (n=78) and Dryas iulia (n=63) used in the long-term memory and reversal learning experiments.](image-url)
Table 5.1 Pairwise comparisons between species in naïve preference between purple and yellow feeders. Bottom left shows z-ratio and top right the associated P-value, corrected for multiple comparisons using Tukey’s test. Asterisks indicate significant pairwise differences.

<table>
<thead>
<tr>
<th></th>
<th>H. erato</th>
<th>H. melpomene</th>
<th>H. hecale</th>
<th>A. vanillae</th>
<th>D. phaetusa</th>
<th>D. iulia</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. erato</td>
<td></td>
<td>0.0001***</td>
<td>&lt;0.0001***</td>
<td>0.7479</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>H. melpomene</td>
<td>4.623***</td>
<td>0.992</td>
<td>0.0016</td>
<td>0.999</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td>H. hecale</td>
<td>5.267***</td>
<td>-0.580</td>
<td>0.0001***</td>
<td>0.999</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>A. vanillae</td>
<td>-1.365</td>
<td>3.864***</td>
<td>4.604***</td>
<td>0.0001***</td>
<td>0.0001***</td>
<td></td>
</tr>
<tr>
<td>D. phaetusa</td>
<td>-5.222***</td>
<td>-0.252</td>
<td>0.375</td>
<td>4.557</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>D. iulia</td>
<td>-5.204***</td>
<td>-0.407</td>
<td>0.192</td>
<td>4.532</td>
<td>-0.180</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2 Naïve colour preference for each species. Results for each species for generalised linear mixed models including only ID as a random effect. P-values less than 0.05 indicate significant deviation from 50%.

|            | z value | Pr(>|z|) |
|------------|---------|---------|
| H. erato   | 6.871   | <0.0001*** |
| H. melpomene| 0.526   | 0.599   |
| H. hecale  | -0.287  | 0.774   |
| A. vanillae| 913.7   | <0.0001*** |
| D. phaetusa| 0.23    | 0.818   |
| D. iulia   | -0.028  | 0.978   |

ii) Interspecific variation in associative colour-learning performance

There were significant differences between species in their fidelity to the trained colour during the first training test (Figure 5.4, $\chi^2=25.317$, d.f.=5, P=0.0001), with Dryadula phaetusa significantly less accurate than all three Heliconius species and Agraulis vanillae (Figure 5.4, Table 5.3). Overall, Heliconius individuals were significantly more accurate than non-Heliconius species (Figure 5.4, $\chi^2=14.608$, d.f.=1, P=0.0001). Nevertheless, all species displayed a high fidelity to the trained colour, with the mean proportion of correct attempts ranging from 0.831 in Dryas iulia to 0.918 in H. hecale (Figure 5.4).
Chapter 5 – Long-term memory and reversal learning ability in Heliconiini butterflies

Figure 5.4 Trained colour preference between purple and yellow for three *Heliconius* species (*H. erato* (n=47), *H. melpomene* (n=48) and *H. hecale* (n=53)) and three non- *Heliconius* Heliconiini, *Agraulis vanillae* (n=80), *Dryadula phaetusa* (n=78) and *Dryas iulia* (n=59) used in the long-term memory and reversal learning experiments.

Table 5.3 Pairwise comparisons between species in fidelity to the trained colour cue during the first recall test. Bottom left shows z-ratio and top right the associated P-value, corrected for multiple comparisons using Tukey’s test. Asterisks indicate significant pairwise differences.

<table>
<thead>
<tr>
<th></th>
<th><em>H. erato</em></th>
<th><em>H. melpomene</em></th>
<th><em>H. hecale</em></th>
<th><em>A. vanillae</em></th>
<th><em>D. phaetusa</em></th>
<th><em>D. iulia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. erato</em></td>
<td></td>
<td>1.0000</td>
<td>0.999</td>
<td>0.962</td>
<td>0.0157*</td>
<td>0.137</td>
</tr>
<tr>
<td><em>H. melpomene</em></td>
<td>-0.084</td>
<td></td>
<td>1.0000</td>
<td>0.950</td>
<td>0.0112*</td>
<td>0.110</td>
</tr>
<tr>
<td><em>H. hecale</em></td>
<td>-0.265</td>
<td>0.180</td>
<td></td>
<td>0.853</td>
<td>0.0033**</td>
<td>0.0522</td>
</tr>
<tr>
<td><em>Agraulis vanillae</em></td>
<td>-0.829</td>
<td>-0.925</td>
<td>-1.166</td>
<td>0.0434*</td>
<td></td>
<td>0.361</td>
</tr>
<tr>
<td><em>Dryadula phaetusa</em></td>
<td>-3.229*</td>
<td>-3.330*</td>
<td>-3.670**</td>
<td>2.899*</td>
<td></td>
<td>0.982</td>
</tr>
<tr>
<td><em>Dryas iulia</em></td>
<td>-2.457</td>
<td>-2.551</td>
<td>-2.834</td>
<td>1.968</td>
<td>0.704</td>
<td></td>
</tr>
</tbody>
</table>
iii) Heliconius show superior visual long-term memory than outgroup Heliconiini

Overall, Heliconius individuals exhibited greater accuracy than non-Heliconius individuals in both the initial trained test (Figure 5.4, z ratio=-2.068, d.f.=inf, P=0.0387) and the long-term memory test (Figure 5.5(a), z ratio=-4.774, d.f.=inf, P<0.0001). Furthermore, non-Heliconius individuals exhibited a significantly larger drop in accuracy between the initial trained test and the long-term memory test than Heliconius individuals (Figure 5.5(a), χ²=5.3094, d.f.=1, P=0.0212).

All species showed a significant shift in preference towards the trained colour after four days of training (Figure 5.5(a), Table 5.4, Table 5.5). Apart from H. melpomene, all species exhibited a significant decline in the fidelity of that preference after the eight-day long-term memory waiting period (Table 5.5). Both H. erato and H. melpomene were significantly more accurate than the three non-Heliconius species during the long-term memory test (Table 5.6). The long-term memory performance of H. hecale was significantly different for Dryas iulia, but not the other non-Heliconius species or the other Heliconius, suggesting a degree of intermediacy or increased statistical variation (Table 5.6). Indeed, uncorrected pairwise comparisons show H. hecale as both performing significantly better than both Dryas iulia and Agraulis vanillae, and worse than the other Heliconius (Table 5.6). The three outgroup Heliconiini, Dryas iulia, Dryadula phaetusa and Agraulis vanilla, did not differ from each other in long-term memory performance (Table 5.6). After a further four days of exposure to white feeders (13 days total after training finished), H. melpomene continued to show a significant preference towards the learned colour, but the non-Heliconius species did not (Figure 5.5(a), Table 5.7), while H. hecale exhibited a preference for the learned colour that approached statistical significance (Figure 5.5(a), Table 5.7).
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Figure 5.5 (a) Long-term memory and (b) reversal learning performance in six Heliconiini species, based on trained food-colour associations. LTM = testing conducted after eight days feeding on white feeders, deprived of the learned stimuli; LTM2 = testing conducted after an additional four days on white feeders; RL1 = testing after the initial reversal of the training paradigm; RL2 = testing after a second reversal back to the original training paradigm. *H. erato* and *Dryas iulia* individuals were dissected for the study presented in Chapter 6 immediately after their LTM trial.
Table 5.4 Analysis of deviance table (Type II Wald chi-square tests) for a generalised linear mixed model for the long-term memory trials. ID and observation level random effects were also included.

<table>
<thead>
<tr>
<th></th>
<th>$\chi^2$</th>
<th>Df</th>
<th>Pr($&gt;\chi^2$)</th>
</tr>
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<tbody>
<tr>
<td>Trial</td>
<td>197.990</td>
<td>1</td>
<td>&lt;0.0001***</td>
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<tr>
<td>Species</td>
<td>38.980</td>
<td>5</td>
<td>&lt;0.0001****</td>
</tr>
<tr>
<td>Trial:Species</td>
<td>12.049</td>
<td>5</td>
<td>0.0341*</td>
</tr>
</tbody>
</table>

Table 5.5 Pairwise comparisons for within species shifts in colour preferences across trials during the long-term memory assay, corrected for multiple comparisons using Tukey’s test. LTM = testing after the eight-day long-term memory period.

<table>
<thead>
<tr>
<th>Species</th>
<th>Contrast</th>
<th>z ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. erato</em></td>
<td>Naïve – Trained</td>
<td>-13.11</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – LTM</td>
<td>4.121</td>
<td>0.0022**</td>
</tr>
<tr>
<td><em>H. melpomene</em></td>
<td>Naïve – Trained</td>
<td>-9.783</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – LTM</td>
<td>2.626</td>
<td>0.265</td>
</tr>
<tr>
<td><em>H. hecale</em></td>
<td>Naïve – Trained</td>
<td>-13.709</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – LTM</td>
<td>6.271</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>Agraulis vanillae</em></td>
<td>Naïve – Trained</td>
<td>-14.996</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – LTM</td>
<td>10.163</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>Dryadula phaetusa</em></td>
<td>Naïve – Trained</td>
<td>-9.949</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – LTM</td>
<td>5.587</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>Dryas iulia</em></td>
<td>Naïve – Trained</td>
<td>-12.338</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – LTM</td>
<td>8.189</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>

Table 5.6 Pairwise comparisons between species in recall accuracy during the long-term memory test. Top right shows P-values corrected for multiple comparisons using Tukey’s test. Bottom left shows uncorrected P-values. Asterisks indicate significant pairwise differences.

<table>
<thead>
<tr>
<th></th>
<th><em>H. erato</em></th>
<th><em>H. melpomene</em></th>
<th><em>H. hecale</em></th>
<th><em>A. vanillae</em></th>
<th><em>D. phaetusa</em></th>
<th><em>D. iulia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. erato</em></td>
<td>1.0000</td>
<td>0.2600</td>
<td>0.0002***</td>
<td>0.0022**</td>
<td>&lt;0.0001***</td>
<td></td>
</tr>
<tr>
<td><em>H. melpomene</em></td>
<td>0.9766</td>
<td>0.425</td>
<td>0.0014**</td>
<td>0.0088**</td>
<td>0.0001***</td>
<td></td>
</tr>
<tr>
<td><em>H. hecale</em></td>
<td>0.0199*</td>
<td>0.0362*</td>
<td>0.258</td>
<td>0.662</td>
<td>0.0176*</td>
<td></td>
</tr>
<tr>
<td><em>Agraulis vanillae</em></td>
<td>&lt;0.0001***</td>
<td>0.0001***</td>
<td>0.0197*</td>
<td>1.0000</td>
<td>0.998</td>
<td></td>
</tr>
<tr>
<td><em>Dryadula phaetusa</em></td>
<td>0.0001***</td>
<td>0.0006***</td>
<td>0.0697</td>
<td>0.783</td>
<td>0.989</td>
<td></td>
</tr>
<tr>
<td><em>Dryas iulia</em></td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
<td>0.0012**</td>
<td>0.338</td>
<td>0.262</td>
<td></td>
</tr>
</tbody>
</table>
**Table 5.7** Colour preference for each species 13 days after training ended. Results for each species for generalised linear mixed models including only ID as a random effect. P-values less than 0.05 indicate significant deviation from 50% preference between purple and yellow.

| Species                | z value | Pr(>|z|)   |
|------------------------|---------|------------|
| *H. melpomene*         | 3.745   | 0.00018*** |
| *H. hecale*            | 1.824   | 0.0682     |
| *Agraulis vanillae*    | -0.593  | 0.553      |
| *Dryadula phaetusa*    | -0.82   | 0.412      |

**iv) Heliconiini butterflies learn reversed colour associations**

All species significantly shifted their colour preference to the rewarded colour after the initial training period and both reversal learning periods (Figure 5.5(b), Table 5.8, Table 5.9). However, when comparing *Heliconius* and non-*Heliconius* individuals, there was a significant clade × trial interaction, indicating overall *Heliconius* are responding differently to reversal training (Table 5.10). The accuracy of *Heliconius* individuals is significantly higher than the non-*Heliconius* during both the first trained test and the second reversal learning test (Table 5.11). However, this difference is not robust when correcting for multiple comparisons (Table 5.11). At the species level, a significant trial × species interaction suggests differences between species in their response to reversal training (Table 5.8). However, pairwise comparisons show that the only significant interspecific differences during either reversal learning tests was *H. erato* outperforming *Dryadula phaetusa* during the first reversal learning test (z-ratio=4.365, P=0.0012).

**Table 5.8** Analysis of deviance table (Type II Wald chi-square tests) for a generalised linear mixed model for the reversal learning trials. ID and observation level random effects were also included.

<table>
<thead>
<tr>
<th></th>
<th>( \chi^2 )</th>
<th>Df</th>
<th>Pr(&gt;( \chi^2 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>1179.935</td>
<td>3</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Species</td>
<td>19.465</td>
<td>5</td>
<td>0.00157**</td>
</tr>
<tr>
<td>Trial:Species</td>
<td>42.944</td>
<td>15</td>
<td>0.00016***</td>
</tr>
</tbody>
</table>
Table 5.9 Pairwise comparisons for within species shifts in colour preferences across trials during the reversal learning assay, corrected for multiple comparisons using Tukey’s test. RL1 = testing after the initial reversal of the training paradigm; RL2 = testing after a second reversal back to the original training paradigm.

<table>
<thead>
<tr>
<th>Species</th>
<th>Contrast</th>
<th>z ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. erato</em></td>
<td>Naïve – Trained</td>
<td>-10.659</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – RL1</td>
<td>12.658</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>RL1 – RL2</td>
<td>-10.165</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>H. melpomene</em></td>
<td>Naïve – Trained</td>
<td>-11.779</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – RL1</td>
<td>13.025</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>RL1 – RL2</td>
<td>-10.329</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>H. hecale</em></td>
<td>Naïve – Trained</td>
<td>-8.117</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – RL1</td>
<td>9.745</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>RL1 – RL2</td>
<td>-7.578</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>Agraulis vanillae</em></td>
<td>Naïve – Trained</td>
<td>-13.478</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – RL1</td>
<td>14.830</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>RL1 – RL2</td>
<td>-10.260</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>Dryadula phaetusa</em></td>
<td>Naïve – Trained</td>
<td>-12.365</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – RL1</td>
<td>14.114</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>RL1 – RL2</td>
<td>-9.636</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>Dryas iulia</em></td>
<td>Naïve – Trained</td>
<td>-7.961</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trained – RL1</td>
<td>9.796</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>RL1 – RL2</td>
<td>-8.517</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>

Table 5.10 Analysis of deviance table (Type II Wald chi-square tests) for a generalised linear mixed model for the reversal learning trials. Species, ID and observation level random effects were also included.

<table>
<thead>
<tr>
<th></th>
<th>χ²</th>
<th>Df</th>
<th>Pr(&gt;χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>1146.546</td>
<td>3</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>Heliconius</em></td>
<td>0.879</td>
<td>1</td>
<td>0.348</td>
</tr>
<tr>
<td>Trial: <em>Heliconius</em></td>
<td>20.764</td>
<td>3</td>
<td>0.00012***</td>
</tr>
</tbody>
</table>

Table 5.11 Pairwise comparisons between *Heliconius* and non-*Heliconius* for each trial during the reversal learning assay, showing both raw p-values and p-values corrected for multiple comparisons using Tukey’s test. RL1 = testing after the initial reversal of the training paradigm; RL2 = testing after a second reversal back to the original training paradigm.

<table>
<thead>
<tr>
<th>Trial</th>
<th>z ratio</th>
<th>Uncorrected P value</th>
<th>Corrected P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>-13.111</td>
<td>0.9095</td>
<td>0.9995</td>
</tr>
<tr>
<td>Trained</td>
<td>4.121</td>
<td>0.0157*</td>
<td>0.0743</td>
</tr>
<tr>
<td>RL1</td>
<td>-9.783</td>
<td>0.1673</td>
<td>0.5113</td>
</tr>
<tr>
<td>RL2</td>
<td>2.626</td>
<td>0.0421*</td>
<td>0.1762</td>
</tr>
</tbody>
</table>
5.4 Discussion

I conducted comparative behavioural experiments testing visual long-term memory and reversal learning ability across three Heliconius species and three outgroup Heliconiini. Based on their expanded mushroom bodies (Chapter 2) and the apparent ecological importance of this cognitive abilities for trapline foraging behaviour, I expected that Heliconius would outperform the other species in both tasks.

Overall, Heliconius exhibited superior long-term retention of the learned colour association compared to the non-Heliconius species (Figure 5.5(a)). Furthermore, although non-Heliconius individuals showed lower fidelity to the learned cue during the first test after training, they also exhibited a significantly larger relative decay of the learned association when tested eight days later – a time period which likely has direct ecological relevance during foraging [133,143]. Importantly, although H. erato and Agraulis vanilla showed a strong bias towards purple (Figure 5.3), this does not appear to have limited their ability to learn a novel colour association, or, in the case of H. erato remember that association (Figure 5.5(a)).

The performance of Heliconius in this task, relative to the closely related outgroup Heliconiini, is consistent with variation in mushroom body size being associated with improved visual long-term memory. Although the role of the mushroom bodies in the formation and maintenance of olfactory long-term memories is well established [88,96,341], the present result is consistent with the mushroom bodies also playing a significant role in visual long-term memory, for at least certain groups of insects. My finding here thus adds to mounting evidence that indicates substantial variation in mushroom body function between insect groups across differing ecologies, reflected in the relative importance of visual and olfactory neural input [83,86]. In Heliconius, mushroom body expansion is predominantly driven by increased visual, rather than olfactory input (Couto et al, in prep.), and the present result demonstrates a possible behavioural consequence of this neural elaboration.

However, it is possible that the improved long-term memory of Heliconius may be restricted to visual information only. This could be tested by conducting the long-term memory experiment using olfactory instead of visual cues. Nevertheless, traplining in Heliconius appears dependent on an enhanced capacity for visual long-term memory, and this result is thus consistent with the mushroom body expansion of Heliconius being driven by the cognitive demands of traplining for pollen. In Drosophila, long-term memory has been specifically
linked to the vertical lobes within the mushroom bodies [96], and we could accordingly expect an expansion of homologous structures in *Heliconius*. However, I cannot currently test this prediction as the lobes in *Heliconius* present as a homogenous unit, making it difficult to distinguish specific sub-regions within the lobes.

Interestingly, the visual long-term memory retention of *H. hecale* appears to be intermediate between *H. erato* and *H. melpomene*, and the outgroup Heliconiini, not being significantly different from any of the tested species. The performance *H. hecale* here is, however, consistent with a concurrently-run study investigating the effects of senescence on long-term memory (Foley, *in prep.*), using independent samples of *H. hecale* and *Dryas iulia*. Importantly, that study compared the long-term memory ability of *H. hecale* with *Dryas iulia* over a larger sample size, and found that *H. hecale* did indeed significantly outperform *Dryas iulia*. In addition, uncorrected pairwise comparisons of long-term memory performance in the present study show *H. hecale* as performing significantly better than *Agraulis vanillae* and *Dryas iulia*, but significantly worse than *H. erato* and *H. melpomene*, while the comparison with *D. dryadula* approaches statistical significance (Table 6). It seems likely, therefore, that the performance of *H. hecale* in this task is truly intermediate between *H. erato* and *H. melpomene* and the outgroup Heliconiini, but nevertheless significantly better than non-pollen feeding Heliconiini. Interestingly, *H. hecale* also performed worse than *H. erato* and *H. melpomene* in a shape-learning task (see Chapter 4). The comparatively poorer performance of *H. hecale* in these tasks is not readily explained by any available neuroanatomical data, which show that the mushroom body size of *H. hecale* is comparable to other *Heliconius*, including *H. erato* and *H. melpomene* (see Chapter 3). There may be a possible ecological explanation, which would mean this result could reflect a slight difference in behavioural response to the cage environment. For example, *H. hecale* is the only species in this study that is predominantly found in the intermediate transition between forest edge and inner forest environments, while the rest are forest edge specialists [137].

I also compared reversal learning performance across the six species, as efficient traplining in *Heliconius* potentially depends on updating established routes in response to changing conditions [142]. Contrary to my predictions, *Heliconius* did not show a clear increase in performance in the reversal learning task compared with the outgroup Heliconiini. Although there is some evidence indicating *Heliconius* overall performed better in the second reversal learning test, a similar disparity was also observed in the initial trained test. This result could thus be attributed simply to a difference in associative learning accuracy, rather than
reversal learning specifically. Currently, the present results do not suggest that *Heliconius* have a superior reversal learning ability than the outgroup Heliconiini. This is somewhat surprising given the apparent importance of the mushroom bodies for reversal learning in honeybees [100,101]. Amongst Lepidoptera, visual reversal learning has been demonstrated in Monarch butterflies, which were able to learn reversed colour cues in as little as two-days [339]. In the present experiment, the training period for each reversal was four days, a duration chosen for its relevance to *Heliconius* traplining behaviour, making the task comparatively easier. It is thus possible that *Heliconius* do learn reversed cues more quickly than the non-*Heliconius* Heliconiini, but this would require testing in a separate experiment.

One caveat associated with this study is that the shift in *Heliconius* mushroom body size, and acquisition of pollen feeding, likely only occurred once (Chapter 2). As such the three *Heliconius* species do not represent independent tests of an association between mushroom body expansion and improved long-term memory. However, many studies on comparative cognition include only one or a few species, which are often distantly related, or often, in the case of vertebrates, consider only a few individuals per species [4]. In the present study, I aimed to balance the inclusion of a representative spread of Heliconiini species with robust sample sizes per species. As such, the differences detected here between the *Heliconius* and non-*Heliconius* species can likely be generalised across the tribe.

In conclusion, the present results show *Heliconius* to possess a superior capacity for retaining learned visual cues, compared with closely related, non-*Heliconius* species, although there were no differences in reversal learning performance. This is the first experimental evidence suggesting that mushroom body expansion in *Heliconius* is associated with cognitive shifts, positioning *Heliconius* as an important example of an enhancement in a specific cognitive ability, over a relatively small phylogenetic scale, co-occurring with a novel foraging strategy. The present findings are highly suggestive of the mushroom body expansion of *Heliconius* as facilitating improved long-term memory in *Heliconius*, plausibly driven by the cognitive demands of trapline foraging. This provides an avenue for exploring the neural mechanisms facilitating this ability to further understand the behavioural consequences of mushroom body expansion. In Chapter 6, I present neuroanatomical data collected from the *H. erato* and *Dryas iulia* individuals that were involved in these long-term trials. There, I investigate the potential neural underpinnings of *Heliconius’* improved long-term memory performance and whether the mushroom bodies of *H. erato* and *Dryas iulia* differ in their plastic response to learning. Future studies could explore this even further through
experimental impairment of the mushroom bodies using, for example, procaine as has been done with honeybees and ants [98,99,101]. Such work would provide confirming evidence for a role of *Heliconius* mushroom bodies in consolidating and maintaining visual long-term memories.
Enhanced visual learning and memory in *Heliconius erato* is associated with increased mushroom body plasticity

6.1 Introduction

There is significant variation in cognitive ability throughout the animal kingdom, both between and within species, yet the neural traits determining these differences are only partly understood, particularly in an evolutionary context [342]. A major strand of research has focused on linking measures of “intelligence” to brain size [1–3]. Indeed, several studies have found correlations between brain size and specific cognitive abilities [5–10,331]. However, as discussed in Chapter 1, attempts to link brain size to cognition, particularly in an interspecific context can be undermined by several factors and have been widely criticised [4,11–13]. Briefly, the use of whole brain size can ignore variation between different regions [20,21], non-linear and non-uniform scaling relationships [14–16], and differences in the underlying structure of the brain such as neuron number [22,23] and connectivity [4,11,12]. In addition, comparative behavioural studies often ignore important ecological differences between species, casting doubt over the biological relevance of the assessed cognitive tasks [4,39,41]. Many of the issues raised above can be avoided by conducting studies comparing individuals within a species, or from closely related species, focusing on linking specific brain regions, and their underlying neural mechanisms, to certain cognitive abilities [44].

A number of studies, primarily looking at intraspecific variation, have found links between specific cognitive abilities and the size or underlying architecture of particular brain regions [74,80,81,90,216,343]. Perhaps one of the most thoroughly explored examples, is the function of the hippocampus in learning and memory in birds and mammals, including many interspecific comparative studies [74–78,344–347]. One major strand of research has centred on the expansion of the hippocampus in food-storing birds [17,72–76], which has been linked to enhanced spatial memory [77–82]. Amongst vertebrates, differences in adult neurogenesis
have also been suggested as a factor in varying cognitive ability [346,348–352]. In insects, however, while experience-dependent adult neurogenesis has been identified in a handful of species [353–356], it is absent entirely in honeybees [357], perhaps the most widely used insect model for studying the neural basis of advanced cognitive abilities [358]. This suggests that neurogenesis is not necessary for higher order cognition in all animal groups. Importantly, synaptic plasticity is also considered to play a key role in learning and memory [359–362]. Yet, there have been few comparative studies investigating interspecific differences in synaptic reorganisation related to experience, and how this may facilitate cognitive evolution [363].

In insects, the mushroom bodies have received a considerable attention as centres of visual, olfactory, and spatial learning and memory [83]. Earlier, in Chapter 1, I provided a brief description of mushroom body structure, which consists of a calyx, the lobes and the peduncle connecting them [86]. The calyces, the subject of this study, are formed by intrinsic neurons called Kenyon cells [84] and receive olfactory and/or visual inputs from the antennal and optic lobes, respectively [86]. These connections between the Kenyon cells and sensory projection neurons form synaptic complexes called microglomeruli [87]. Notably, the mushroom bodies exhibit a large degree of developmental and experience-related plasticity. Microglomeruli density and number can vary with both age and experience, and has been linked with memory formation in Hymenoptera [88–92,232]. In conjunction with these changes in cellular growth and synaptic density, the mushroom body calyx can also undergo posteclosion volumetric expansion [215,216,232,364,365]. Although absent in honeybees [357], adult Kenyon cell neurogenesis has been shown in several insects [353,356], including an example from the Lepidoptera, the moth *Agrotis ipsilon* [354]. In the cricket *Acheta domesticus*, blocking of mushroom body neurogenesis led to impaired learning and memory [355]. As highly plastic structures in terms of both organisation and overall volume, the mushroom bodies are an excellent model for investigating the neural underpinnings of cognitive variation. However, few studies have investigated links between mushroom bodies and behaviour in an interspecific, comparative framework [21,215,363,366].

*Heliconius* butterflies are an excellent system for investigating the neural underpinnings of improved cognitive ability, through investigation of the mushroom body. Compared with closely related Heliconiini, *Heliconius* exhibit an enhanced visual, long-term memory (Chapter 5), likely important for their trapline foraging behaviour. The superior long-term memory of *Heliconius* may be associated not just with their marked expansion of the mushroom bodies (Chapter 2), but also potentially with changes in structure and plasticity that may co-evolve
with mushroom body size. Marked posteclosion growth of the calyx has been identified in *Heliconius*, with wild-caught individuals also exhibiting larger mushroom bodies than captive reared butterflies, potentially in response to foraging experience [129]. However, the causes and developmental mechanisms responsible for this growth is unknown. Here, I investigate how the plasticity of the mushroom body calyx varies between *Heliconius erato* and the Heliconiini *Dryas iulia*, in response to aging and learning. These two species are closely related, having diverged approximately 27 million years ago [131], and, with the exception of *Heliconius*’ novel pollen feeding foraging strategy, show a broad ecological similarity, making them ideally suited for comparative behavioural and neuroanatomical study.

To test for the effects of learning on mushroom body plasticity, I compared individuals trained to make food-colour associations against control individuals raised in a “non-learning” environment where colours were equally rewarded and punished. I also investigated the effects of aging by making further comparisons with freshly eclosed butterflies. Individuals were compared across several traits in the mushroom body calyx – synapse density and total number, total Kenyon cell number and calyx volume. Finally, I investigated specific neural correlates of long-term memory by testing for within-species associations between recall accuracy and measured neural traits. To my knowledge, this study is the first to directly test for interspecific variation in the effects of age and learning experience on the size and structural organisation of the mushroom body calyx. These results will provide valuable insight into the neural underpinnings of both inter- and intraspecific variation in cognitive ability.

### 6.2 Methods

**i) Butterfly treatment groups**

*Dryas iulia* and *Heliconius erato* butterflies were reared from stock populations established using locally-caught, wild butterflies, at the Smithsonian Tropical Research Institute in Gamboa, Panama. Caterpillars from both species were reared on *Passiflora biflora* in mesh pop-up cages outdoors in ambient conditions. After eclosing, adults were divided into three treatment groups – Day 0, Learning and Control (Figure 6.1). Day 0 butterflies comprised individuals that were dissected the evening of their day of emergence. These butterflies were kept in a small, mesh pop-up cage until dissection and had no foraging or free-flight experience. The Learning group comprised the *Heliconius erato* and *Dryas iulia* individuals that completed the long-term memory assay described in Chapter 5, which sets out their training and testing
Learning group individuals were sacrificed for dissection the day they finished their long-term memory preference test, 17 days after eclosing. The Control group consisted of individuals aged-matched to the Learning group reared in a “non-learning” environment. Control individuals were acclimatised to the use of artificial feeders and then tested for naïve colour preference following the same protocol used for the Learning group (Chapter 5). These butterflies were then introduced to a “training” cage for four days, which contained an even number of purple and yellow feeders, presented in random spatial arrangement. However, unlike the Learning group, where feeder colour was consistently associated with either a food reward or quinine punishment, half of the feeders for each colour were filled with the rewarding sugar-protein mixture, and half with the aversive quinine solution. Each colour, therefore, was as equally rewarding as punishing. After four days in this environment, colour preference was again tested following the method in Chapter 5. Butterflies were then introduced to a cage with white feeders for eight days, replicating the long-term memory waiting period of the Learning group (Chapter 5). Finally, after eight days feeding on white feeders, Control butterflies were exposed to empty purple and yellow feeders from 08:00 to 12:00 on their final morning, mirroring the last preference test of the Learning group, before being dissected in the evening.

Figure 6.1 Schematic of the three treatment groups for Dryas iulia and Heliconius erato, showing feeder experience, testing, and day of dissection.

ii) **Brain dissection, fixing and training**

All brains were dissected and fixed at the Smithsonian Tropical Research Institute in Gamboa, Panama, following established protocols [129,233]. Butterflies were decapitated using scissors and the head was submerged under HEPES-buffered saline (HBS; 150 mM NaCl; 5 mM KCl; 5 MM CaCl$_2$; 25 mM sucrose; 10 mM HEPES; pH 7.4). A small aperture was cut into the
head cuticle between the eyes to improve permeation of the fixative. The brain was fixed \textit{in situ} for 16-20 hours at room temperature under gentle agitation in zinc-formaldehyde solution (ZnFA; 0.25% [18.4 mM] ZnCl$_2$; 0.788% [135 mM] NaCl; 1.2% [35 mM] sucrose; 1% formaldehyde). After fixing, the whole brain was dissected out under HBS using a scalpel and forceps, placed into 80% methanol, 20% dimethyl sulfoxide (DMSO) under agitation for 2 hours and then transferred to 100% methanol for long-term storage at -20°C.

Brains were subject to three types of staining. Anti-synapsin was used to mark synapses in the calyx [88,90,367]. I used DAPI to identify nuclei in Kenyon cell bodies [233,368], along with HRP (horseradish peroxidase) to label neuron membranes to confirm that counted nuclei were neuronal [369]. Brains were stained in batches of eight that included individuals from all treatment groups to avoid the possibility of batch effects skewing results. Prior to staining, brains were first rehydrated in a decreasing methanol series (90%, 70%, 50%, 30%, 0% in 0.1 M Tris buffer, pH 7.4) for 10 minutes each. Because quantification of the Kenyon cells and synapses requires imaging the brain at 63X magnification, it was necessary to section the brains for calyx tissue to be within the working distance of the objective lens of the microscope. Brains were embedded in 5% agarose which was cut into a rectangular prism. The agarose block was cut along a corner so that individual slices could be correctly orientated later during mounting. The embedded brain was submerged in 0.1 M Tris buffer and sliced horizontally into 80 μm sections using a Leica VT1000 S vibrating blade microtome.

After sectioning, brain slices were permeabilised in PBSd-NGS (1% DMSO, 5% normal goat serum (NGS) diluted in 0.1 M phosphate-buffered saline (PBS; 7.4 pH)) for two hours. A mouse antibody targeting synapsin (monoclonal Anti-SYNORF1; 3C11; [1:30]) and rabbit HRP [1:5000] were diluted in PBSd-NGS, then applied at a 1:30 dilution in PBSd-NGS and kept for three days at 4°C under low agitation. Samples were then washed in PBS (3 x 2 hours) before applying the Cy2-conjugated anti-mouse and Cy3-conjugating anti-rabbit antibodies, at 1:100 and 1:200, respectively, in PBSd-NGS. Samples were then kept at 4°C under low agitation for a further three days. Samples were then rinsed in PBS (3 x 2 hours). In preparation for the DAPI stain, samples were washed in 0.2% Triton in distilled H$_2$O for 10 minutes. DAPI was applied 1:1000 in 0.2% Triton and H$_2$O under agitation for 30 minutes at room temperature. After, samples were washed in 0.2% Triton and H$_2$O for 10 minutes and then in 0.2% Triton and PBS (4 x 10 minutes) and then placed overnight in 60% glycerol in PBS. Brain sections were then mounted in 80% glycerol on slides under a coverslip sealed with nail polish and stored in the dark. Due to the delicate nature of the 80 μm slices, samples from
some individuals suffered physical damage during staining and mounting meaning that their total calyx volume, and synapse and Kenyon cell counts could not be reconstructed.

**iii) Confocal microscopy and image processing**

All brains were imaged using a laser-scanning confocal microscope (Upright Leica SP5, Leica Microsystems, Mannheim, Germany), at a resolution of 1024x1024 pixels. Mushroom body calyces were scanned using 10X dry objective (0.4 NA), with a mechanical z-step of 1 μm, with each brain section scanned individually. Kenyon cells and synapses were imaged using the 63X objective (1.3 NA) under a glycerol immersion, with a mechanical z-step of 1 μm. For each individual, five regions of mushroom body calyx and Kenyon cell cluster were chosen at random for scanning. Cy2 was excited with the Argon laser at 488 nm. The solid-state laser was used to excite DAPI at 405 nm and Cy3 at 561 nm. Wavelengths were scanned sequentially.

Brain images were processed using ImageJ v 1.53n and Amira 3D 2021.2. Calyx volumes were reconstructed using Amira, with each brain section segmented separately. For each stack, every third or fourth image was manually segmented by highlighting the region covered by the calyx and then interpolated across the z-dimension (Figure 6.2). The *measure statistics* function was used to extract volumes (in μm$^3$) for each section, which were later added together for the total calyx volume and a correction factor of 2.0421 was applied. Synapse densities were estimated using ImageJ. The *3D Objects Counter* function was used to automatically count 3D objects within five 50x50x15 μm boxes (Figure 6.3(a),(b)). For each scan, the brightness threshold was adjusted manually so that only distinct objects were counted. To reduce noise, objects smaller than 10 voxels were not counted. The total number of synapses in the calyx was then estimated by calculating the average synapse density across the five scans and multiplying it by the total calyx volume, assuming homogeneity of synapse densities across the calyx. Kenyon cell cluster volumes and total Kenyon cell numbers were determined in a similar manner. Kenyon cell density was estimated from five randomly selected 25x25x15 μm boxes in the cell cluster (Figure 6.3(c),(d)). Cell numbers within each box were automatically counted using the *Modular Image Analysis* (MIA) and *Stardist* plugins in ImageJ [370,371]. *Stardist* detects objects with star-convex shape priors and can be used for detecting cells. Total cell counts were then estimated by multiplying the average density by the total volume of the cell cluster. The Kenon cell data were collected by Amaia Alcalde.
Figure 6.2 Examples of HRP staining of the mushroom body calyx, imaged at 10X using a laser-scanning confocal microscope, in (a) Heliconius erato and (b) Dryas iulia from the Learning group. White dashed line shows the calyx. Red dashed line shows the Kenyon cell cluster. Scale bar represents 200 μm.
iv) Statistical analyses

To determine whether the control individuals had failed to develop a learned colour preference, their feeding choices before and after experience with coloured feeders were analysed using a binomially distributed GLMM using the function `glmmTMB` from the package `glmmTMB` v1.1.2.3 for R [372]. Training and species, and their interaction, were treated as fixed effects with individual and observation-level random effects. Preference shift in the Learning group
was assessed in the same manner. These models, and all following GLMMs, were diagnosed using the R package `DHARMa` v 0.4.4 [294]. All post-hoc comparisons were made by obtaining the estimated marginal means using the package `emmeans` v1.7.0 and were corrected for multiple comparisons using the Šidák test [315].

For statistical analysis, synapse densities and counts were square-root transformed, and calyx volume and Kenyon cell counts were log$_{10}$ transformed to better fit a normal distribution. I then ran a series of GLMMs examining how these neuroanatomical traits varied within species between groups and between species. For these analyses, I removed two individuals from the Learning group, one from each species, whose accuracy was less than 50% during the initial recall test, as I assumed those individuals had not acquired a learned association with the trained colour. For each trait I ran a GLMM with a Gaussian distribution, treating group and species, and their interaction, as fixed effects. I further tested for variation in the scaling relationships between Kenyon cell number and number of synapses in the calyx, and Kenyon cell number and calyx volume, using the `sma` function in the R package `smatr` v 3.4-8 [257]. This analysis allows for the detection of shifts in elevation in the scaling relationship between two traits. The “robust” option was set to true for these analyses and multiple comparisons were corrected for [258].

Finally, I investigated whether specific neural traits correlate with individual recall performance during the long-term memory assay (Chapter 5). For each species, I ran a series of binomial GLMMs, with ID as a random effect, testing whether recall accuracy varied with each trait. These analyses were repeated for both the initial recall test and the long-term recall test.

### 6.3 Results

#### i) Control butterflies show no shift in colour preference

As described and discussed in Chapter 5, Learning group *Dryas iulia* and *Heliconius erato* both significantly shifted their preference towards the colour rewarded during training (Figure 6.4, Tables 6.1 & 6.2), with *Heliconius erato* showing higher accuracy in both initial and long-term recall (Figure 6.4, Table 6.1). In contrast, neither species from the Control group exhibited a shift in colour preference (Figure 6.4, Tables 6.1 & 6.2), suggesting that they did not form a learned association with either colour.
**Figure 6.4** Shifts in colour preference in *Dryas iulia* and *Heliconius erato* for (a),(b) the Learning group and (c),(d) the Control group. For the Learning group, each colour was consistently presented with either a food reward or punishment. For the Control group, both colours had an equal chance of being rewarding or punishing. LTM = long-term memory test after eight days feeding on only white feeders.

**Table 6.1** ANOVA results for a GLMM testing for the effects of training and species, and their interaction, on colour preference for Learning and Control groups in *Dryas iulia* and *Heliconius erato*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Factor</th>
<th>$\chi^2$</th>
<th>Df</th>
<th>Pr($&gt;\chi^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Learning</td>
<td>Trial</td>
<td>197.333</td>
<td>1</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>6.3575</td>
<td>1</td>
<td>0.0117*</td>
</tr>
<tr>
<td></td>
<td>Trial:Species</td>
<td>28.733</td>
<td>1</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Control</td>
<td>Trial</td>
<td>0.106</td>
<td>1</td>
<td>0.750</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>34.2235</td>
<td>1</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Trial:Species</td>
<td>0.7283</td>
<td>1</td>
<td>0.393</td>
</tr>
</tbody>
</table>
Table 6.2 Pairwise comparisons between colour preference before and after experience with coloured feeders for *Dryas iulia* and *Heliconius erato* from the Learning and Control groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Species</th>
<th>t ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Learning</td>
<td><em>Dryas iulia</em></td>
<td>-11.358</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td><em>H. erato</em></td>
<td>-12.071</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Control</td>
<td><em>Dryas iulia</em></td>
<td>0.587</td>
<td>0.916</td>
</tr>
<tr>
<td></td>
<td><em>H. erato</em></td>
<td>2.177</td>
<td>0.652</td>
</tr>
</tbody>
</table>

**ii) Mushroom bodies of *Dryas iulia* and *Heliconius erato* vary in their response to aging and learning**

*Dryas iulia* and *Heliconius erato* varied significantly in how the mushroom body responded to both aging and learning (Figure 6.5, Tables 6.3 & 6.4). In *Heliconius erato*, the Learning and Control individuals had both a decreased synapse density and a lower total number of synapses in the calyx, but increased calyx volumes, relative to Day 0 individuals (Figure 6.5(a)-(c), Table 6.4). I also detected a difference between the Learning and Control groups for *Heliconius erato*, with the Learning group having a higher number of synapses in the calyx than the Control individuals (Figure 6.5(c), Tables 6.3 & 6.4) – a result also reflected in synapses density when considering the uncorrected P-value (Table 6.4). This was true even when controlling for Kenyon cell number (t ratio=4.792, d.f. = 73, P<0.0001), suggesting that *Heliconius erato* individuals in the Learning group have more synapses per Kenyon cell than the Control individuals.

In contrast, for *Dryas iulia*, none of synapse density, calyx volume, synapse number nor Kenyon cell number, varied with age or experience (Figure 6.5, Tables 6.3 & 6.4). This difference between the two species is further reflected in the significant interaction between treatment group and species for synapse density, synapse number and calyx volume. In general, however, the results for *Dryas iulia* do exhibit the same trends as *Heliconius erato* (Figure 6.5). If the Learning and Control groups for *Dryas iulia* are pooled together and the data analysed solely by age, then *Dryas iulia* do exhibit slight, but significant, post-eclosion expansion of the calyx (Figure 6.5(b), $\chi^2$=5.0132, d.f.=1, P=0.0252), although the effect is much smaller than in *Heliconius erato*. Notably, although there was no intraspecific variation between treatment groups in Kenyon cell number (Figure 6.5, Table 6.4), pooling the Learning and Control groups together, while controlling for species, shows a significant age effect (Figure 6.5(d), $\chi^2$=4.4821,
d.f.=1, P=0.0343), suggesting the possibility of a small amount of posteclosion neurogenesis of Kenyon cells.

When comparing *Heliconius erato* and *Dryas iulia* individuals from the same treatment group, *Heliconius erato* have larger calyces and more Kenyon cells across all groups. However, within each treatment group there were no interspecific differences in synapse density (Figure 6.5, Table 6.4). Total synapse count in the calyx is higher in *Heliconius erato* compared to *Dryas iulia* for the Day 0 and Learning groups, but not the Control group (Figure 6.5(c), Table 6.4).

*Figure 6.5* Variation in (a) synapse density in the calyx, (b) mushroom body calyx volume, (c) total synapse number in the calyx, and (d) total Kenyon cell number, between three treatment groups, Day 0, Learning and age-matched Controls, in *Dryas iulia* and *Heliconius erato*. Only within-species statistical comparisons are shown. * = P<0.05; *** = P < 0.0001.
Table 6.3 ANOVA results for a series of GLMMs testing for variation between *Dryas iulia* and *Heliconius erato*, divided into three treatment groups – Day 0, Learning and Control – in several neuroanatomical traits associated with the mushroom bodies.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Factor</th>
<th>χ²</th>
<th>Df</th>
<th>Pr(&gt;χ²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synapse density</td>
<td>Group</td>
<td>36.0732</td>
<td>2</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>1.466</td>
<td>1</td>
<td>0.226</td>
</tr>
<tr>
<td></td>
<td>Group:Species</td>
<td>6.734</td>
<td>2</td>
<td>0.0344*</td>
</tr>
<tr>
<td>Mushroom body calyx volume</td>
<td>Group</td>
<td>32.5205</td>
<td>2</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>831.8497</td>
<td>1</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Group:Species</td>
<td>6.8719</td>
<td>2</td>
<td>0.0322*</td>
</tr>
<tr>
<td>Number of synapses</td>
<td>Group</td>
<td>27.898</td>
<td>2</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>43.219</td>
<td>1</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Group:Species</td>
<td>11.154</td>
<td>2</td>
<td>0.0038**</td>
</tr>
<tr>
<td>Number of Kenyon cells</td>
<td>Group</td>
<td>4.9649</td>
<td>2</td>
<td>0.0835</td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>831.3563</td>
<td>1</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>Group:Species</td>
<td>0.1642</td>
<td>2</td>
<td>0.922</td>
</tr>
</tbody>
</table>
Table 6.4 Selected pairwise comparisons in several neuroanatomical traits associated with the mushroom bodies. Comparisons are made within species between groups, and between species for individuals in the same treatment group. Corrected for multiple comparisons using the Šidák test.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Species</th>
<th>Contrast</th>
<th>t ratio</th>
<th>Uncorrected P value</th>
<th>Corrected P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synapse density</td>
<td>Dryas iulia</td>
<td>Day 0 – Learning</td>
<td>1.246</td>
<td>0.216</td>
<td>0.518</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>1.859</td>
<td>0.0663</td>
<td>0.186</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>0.918</td>
<td>0.361</td>
<td>0.739</td>
</tr>
<tr>
<td></td>
<td>H. erato</td>
<td>Day 0 – Learning</td>
<td>5.267</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>6.589</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>2.131</td>
<td>0.0359*</td>
<td>0.104</td>
</tr>
<tr>
<td></td>
<td>Dryas iulia</td>
<td>Day 0</td>
<td>-1.570</td>
<td>0.120</td>
<td>0.319</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning</td>
<td>1.433</td>
<td>0.155</td>
<td>0.397</td>
</tr>
<tr>
<td></td>
<td>H. erato</td>
<td>Control</td>
<td>1.921</td>
<td>0.0580</td>
<td>0.164</td>
</tr>
<tr>
<td>Mushroom body calyx volume</td>
<td>Dryas iulia</td>
<td>Day 0 – Learning</td>
<td>-2.047</td>
<td>0.0441*</td>
<td>0.127</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>-1.449</td>
<td>0.151</td>
<td>0.389</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>0.360</td>
<td>0.720</td>
<td>0.978</td>
</tr>
<tr>
<td></td>
<td>H. erato</td>
<td>Day 0 – Learning</td>
<td>-5.710</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>-4.482</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>1.140</td>
<td>0.258</td>
<td>0.591</td>
</tr>
<tr>
<td></td>
<td>Dryas iulia</td>
<td>Day 0</td>
<td>-12.728</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning</td>
<td>-20.624</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>H. erato</td>
<td>Control</td>
<td>-15.855</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Number of synapses</td>
<td>Dryas iulia</td>
<td>Day 0 – Learning</td>
<td>0.446</td>
<td>0.657</td>
<td>0.960</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>1.011</td>
<td>0.315</td>
<td>0.679</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>0.721</td>
<td>0.473</td>
<td>0.854</td>
</tr>
<tr>
<td></td>
<td>H. erato</td>
<td>Day 0 – Learning</td>
<td>3.656</td>
<td>0.0005***</td>
<td>0.0014**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>6.158</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>2.805</td>
<td>0.0064**</td>
<td>0.0191*</td>
</tr>
<tr>
<td></td>
<td>Dryas iulia</td>
<td>Day 0</td>
<td>-6.007</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning</td>
<td>-4.022</td>
<td>0.0001</td>
<td>0.0004***</td>
</tr>
<tr>
<td></td>
<td>H. erato</td>
<td>Control</td>
<td>-1.453</td>
<td>0.150</td>
<td>0.387</td>
</tr>
<tr>
<td>Number of Kenyon cells</td>
<td>Dryas iulia</td>
<td>Day 0 – Learning</td>
<td>-1.491</td>
<td>0.140</td>
<td>0.365</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>-1.473</td>
<td>0.145</td>
<td>0.380</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>-0.210</td>
<td>0.835</td>
<td>0.996</td>
</tr>
<tr>
<td></td>
<td>H. erato</td>
<td>Day 0 – Learning</td>
<td>-0.953</td>
<td>0.344</td>
<td>0.718</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>-1.525</td>
<td>0.132</td>
<td>0.345</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>-0.621</td>
<td>0.536</td>
<td>0.900</td>
</tr>
<tr>
<td></td>
<td>Dryas iulia</td>
<td>Day 0</td>
<td>-15.361</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning</td>
<td>-18.646</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td>H. erato</td>
<td>Control</td>
<td>-15.744</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
</tbody>
</table>
I also tested for variation in the scaling relationship between Kenyon cell number and synapse number, and Kenyon cell number and calyx volume. Both scaling relationships showed significant variation both between species and between treatment groups within species (Figure 6.6, Table 6.5). For *Heliconius erato*, Day 0 individuals had significantly more synapses per Kenyon cell than Control individuals, but not those from the Learning group (Figure 6.6(a), Table 6.5). When considering uncorrected P-values, Learning *Heliconius erato* have significantly more synapses per Kenyon cell than their Control counterparts (Figure 6.6(a), Table 6.5). Comparing between species, Learning and Control *Heliconius erato* had fewer synapses per Kenyon cell than *Dryas iulia* from the same groups, although there was no such difference for Day 0 butterflies (Figure 6.6(a), Table 6.5). There was no difference between *Dryas iulia* groups in the relationship between calyx volume and Kenyon cell number (Table 6.5). However, Learning *Heliconius erato* had larger calyx volumes for a given number of Kenyon cells, compared to Day 0 butterflies. The same was true for Control *Heliconius erato* when looking at uncorrected P-values (Table 6.5). No treatment groups, however, showed any interspecific differences in the relationship between Kenyon cell count and calyx volume (Figure 6.6(b), Table 6.5), suggesting the relationship between these two traits is consistent between the two species. This relationship between Kenyon cells and calyx volume is non-isometric, scaling at a slope of 0.802, with larger mushroom bodies having proportionately more Kenyon cells.

![Figure 6.6](image.png) Variations in elevation in the scaling relationships between Kenyon cell number and (a) synapse number in the calyx; and (b) calyx volume for *Dryas iulia* and *Heliconius erato* across three treatment groups.
Enhanced memory in *Heliconius erato* associated with increased neuroplasticity

### Table 6.5 Pairwise comparisons for changes in elevation in the scaling relationship between Kenyon cell number and synapse number in the calyx, and Kenyon cell number and calyx volume. Comparisons made using the R package *smatr*.

<table>
<thead>
<tr>
<th>Regression</th>
<th>Species</th>
<th>Contrast</th>
<th>Test stat</th>
<th>Uncorrected P value</th>
<th>Corrected P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of synapses</td>
<td><em>Dryas iulia</em></td>
<td>Day 0 – Learning</td>
<td>2.83</td>
<td>0.123</td>
<td>0.859</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>5.111</td>
<td>0.0237*</td>
<td>0.303</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>0.568</td>
<td>0.451</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td><em>H. erato</em></td>
<td>Day 0 – Learning</td>
<td>7.431</td>
<td>0.0064**</td>
<td>0.092</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>24.459</td>
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<td>&lt;0.0001***</td>
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<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>4.955</td>
<td>0.0260*</td>
<td>0.327</td>
</tr>
<tr>
<td>Number of Kenyon cells</td>
<td><em>Dryas iulia</em></td>
<td>Day 0</td>
<td>4.866</td>
<td>0.0274*</td>
<td>0.341</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning</td>
<td>26.805</td>
<td>&lt;0.0001***</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>18.143</td>
<td>&lt;0.0001***</td>
<td>0.0003***</td>
</tr>
<tr>
<td></td>
<td><em>H. erato</em></td>
<td>Day 0</td>
<td>0.526</td>
<td>0.468</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning</td>
<td>0.0003</td>
<td>0.986</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>0.0300</td>
<td>0.862</td>
<td>1.000</td>
</tr>
<tr>
<td>Calyx volume</td>
<td><em>Dryas iulia</em></td>
<td>Day 0 – Learning</td>
<td>9.727</td>
<td>0.0018**</td>
<td>0.0268*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0 – Control</td>
<td>5.695</td>
<td>0.0170*</td>
<td>0.227</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning – Control</td>
<td>2.190</td>
<td>0.139</td>
<td>0.894</td>
</tr>
<tr>
<td></td>
<td><em>H. erato</em></td>
<td>Day 0</td>
<td>0.625</td>
<td>0.429</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Learning</td>
<td>0.00288</td>
<td>0.957</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>0.673</td>
<td>0.412</td>
<td>0.999</td>
</tr>
</tbody>
</table>

**iii) Neural correlates of recall performance**

I tested for relationships between performance in the initial recall (16 hours removed from the colour cues) and long-term recall tests (eight days removed) across several neuroanatomical traits in the mushroom body calyx (Figures 6.7 & 6.8, Table 6.6). *Dryas iulia* and *Heliconius erato* differed in the effects measured traits had on recall performance. For *Dryas iulia*, initial recall accuracy was negatively associated with calyx volume (Figure 6.7(b), Table 6.6), whereas for *Heliconius erato* it was positively correlated with synapse density and number in the calyx, and with the ratio of synapses to Kenyon cells (Figure 6.7(g),(i),(k), Table 6.6). For the long-term, eight-day recall, however, accuracy in both species was not correlated with any of the measured neuroanatomical traits (Figure 6.8). In the case of *Heliconius erato*, this result does seem to be influenced by the presence of an outlier individual, which showed a very high synapse count, while scoring very low on the long-term recall test despite high performance in the initial learning trial (Figure 6.8(i),(k)). If this individual is removed, a significant positive association is recovered between long-term recall performance and number of synapses in the calyx (Figure 6.9(a), $\chi^2=9.263$, d.f.=1, $P=0.00234$) and the ratio of synapses to Kenyon cells (Figure 6.9(b), $\chi^2=10.918$, d.f.=1, $P<0.001$).
Chapter 6 – Enhanced memory in *Heliconius erato* associated with increased neuroplasticity

Figure 6.7 Relationship between performance in the initial recall test (Chapter 5) and several neuroanatomical measurements in the mushroom body calyx for (a)-(f) *Dryas iulia* and (g)-(l) *Heliconius erato*. Regression lines, with standard errors, are from GLMM analysis and are shown when the trait was a significant predictor of performance.
Figure 6.8 Relationship between performance in the long-term recall test (Chapter 5) and several neuroanatomical measurements in the mushroom body calyx for (a)-(f) *Dryas iulia* and (g)-(l) *Heliconius erato*. Asterix in (i) and (k) indicates outlier individual.
Table 6.6 ANOVA results for a series of GLMMs testing for relationships between neuroanatomical measurements in the mushroom body calyx and performance in the initial and long-term recall tests in *Dryas iulia* and *Heliconius erato*.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Test</th>
<th>Species</th>
<th>$\chi^2$</th>
<th>Df</th>
<th>Pr($&gt;\chi^2$)</th>
</tr>
</thead>
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<tr>
<td>Synapse density</td>
<td>Initial recall</td>
<td><em>Dryas iulia</em></td>
<td>1.4775</td>
<td>1</td>
<td>0.224</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>H. erato</em></td>
<td>5.4732</td>
<td>1</td>
<td>0.0193**</td>
</tr>
<tr>
<td></td>
<td>Long-term recall</td>
<td><em>Dryas iulia</em></td>
<td>0.4562</td>
<td>1</td>
<td>0.499</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>H. erato</em></td>
<td>0.1482</td>
<td>1</td>
<td>0.700</td>
</tr>
<tr>
<td>Mushroom body calyx volume</td>
<td>Initial recall</td>
<td><em>Dryas iulia</em></td>
<td>7.6747</td>
<td>1</td>
<td>0.0056**</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>H. erato</em></td>
<td>0.676</td>
<td>1</td>
<td>0.411</td>
</tr>
<tr>
<td></td>
<td>Long-term recall</td>
<td><em>Dryas iulia</em></td>
<td>0.0368</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td><em>H. erato</em></td>
<td>0.099</td>
<td>1</td>
<td>0.753</td>
</tr>
<tr>
<td>Number of synapses</td>
<td>Initial recall</td>
<td><em>Dryas iulia</em></td>
<td>0.3431</td>
<td>1</td>
<td>0.558</td>
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<tr>
<td></td>
<td></td>
<td><em>H. erato</em></td>
<td>9.2053</td>
<td>1</td>
<td>0.00241**</td>
</tr>
<tr>
<td></td>
<td>Long-term recall</td>
<td><em>Dryas iulia</em></td>
<td>0.3575</td>
<td>1</td>
<td>0.550</td>
</tr>
<tr>
<td></td>
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<td><em>H. erato</em></td>
<td>1.6108</td>
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<td>0.206</td>
</tr>
<tr>
<td>Number of Kenyon cells</td>
<td>Initial recall</td>
<td><em>Dryas iulia</em></td>
<td>0.0068</td>
<td>1</td>
<td>0.934</td>
</tr>
<tr>
<td></td>
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<td><em>H. erato</em></td>
<td>1.5854</td>
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<td>0.208</td>
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<tr>
<td></td>
<td>Long-term recall</td>
<td><em>Dryas iulia</em></td>
<td>0.4704</td>
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<td>0.493</td>
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<tr>
<td></td>
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<td><em>H. erato</em></td>
<td>1.5167</td>
<td>1</td>
<td>0.218</td>
</tr>
<tr>
<td>Ratio of synapses to Kenyon cells</td>
<td>Initial recall</td>
<td><em>Dryas iulia</em></td>
<td>0.576</td>
<td>1</td>
<td>0.448</td>
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<tr>
<td></td>
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<td><em>H. erato</em></td>
<td>12.852</td>
<td>1</td>
<td>0.00034***</td>
</tr>
<tr>
<td></td>
<td>Long-term recall</td>
<td><em>Dryas iulia</em></td>
<td>0.2323</td>
<td>1</td>
<td>0.630</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>H. erato</em></td>
<td>3.2646</td>
<td>1</td>
<td>0.0708</td>
</tr>
<tr>
<td>Ratio of calyx volume to Kenyon cells</td>
<td>Initial recall</td>
<td><em>Dryas iulia</em></td>
<td>1.8266</td>
<td>1</td>
<td>0.177</td>
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<tr>
<td></td>
<td></td>
<td><em>H. erato</em></td>
<td>0.2405</td>
<td>1</td>
<td>0.624</td>
</tr>
<tr>
<td></td>
<td>Long-term recall</td>
<td><em>Dryas iulia</em></td>
<td>0.3875</td>
<td>1</td>
<td>0.534</td>
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<tr>
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<td></td>
<td><em>H. erato</em></td>
<td>0.3793</td>
<td>1</td>
<td>0.538</td>
</tr>
</tbody>
</table>
Chapter 6 – Enhanced memory in *Heliconius erato* associated with increased neuroplasticity

**Figure 6.9** Long-term recall accuracy in *Heliconius erato*. When a single outlier individual (grey) is removed, (a) total synapse number in the calyx ($\chi^2=9.263$, d.f.=1, $P=0.0023$) and (b) the ratio of synapses to Kenyon cells ($\chi^2=10.918$, d.f.=1, $P<0.001$) show a significant positive relationship with performance in the long-term recall test. Regression lines, with standard errors, are from GLMM analysis.

### 6.4 Discussion

Compared with the closely related Heliconiini, *Dryas iulia*, *Heliconius erato* exhibit both expanded mushroom bodies (Chapter 2) and improved visual long-term memory (Chapter 5). Here, I further explore the neural underpinnings of this improved cognitive ability by investigating whether aging and experience differentially affect the post-eclosion development of the mushroom bodies in these two species. The present results indicate that the mushroom bodies of *Heliconius erato* do, indeed, exhibit greater age- and experience-associated variation than those of *Dryas iulia* (Figures 6.5 & 6.6).

The calyces of *Heliconius erato* show both an increase in total volume and decrease in synapse density with increasing age, whereas *Dryas iulia* show no such effect (Figure 6.5(a),(b)). Age-associated increases in calyx volume and decreases in synapse density have similarly been observed in a number of Hymenoptera including bumblebees [90,232], honeybees [91,373], ants [92,363] and paper wasps [374,375]. Although posteclosion expansion of the calyx is known in other butterflies [215], including another species of *Heliconius*, *H. hecale* [129], the present results are the first report of synapse restructuring in a maturing adult butterfly. I found that the total number of synapses in the calyx decreased with
age and experience in *Heliconius erato*. Honeybees [91,367,373] and desert ants [92] show a similar effect, although synapse number in the calyx was seen to *increase* with age in the ant *Camponotus rufipes* [376]. This “pruning” of synapses is well-recognised method of refining neural connectivity, and is also present in vertebrates [377–380]. During pruning, axonal branches are selectively eliminated, increasing the strength of remaining synaptic connections [377,381,382]. Although showing evidence of slight posteclosion volumetric expansion (Figure 6.5(b), Dryas iulia exhibited no significant age- or experience-related structural changes in the calyx (Figure 6.5). This indicates that the mushroom bodies of *Heliconius erato* are not just larger, but also more plastic. *Heliconius erato* mushroom bodies exhibit a greater restructuring in response to experience than *Dryas iulia*, potentially facilitating superior visual learning and memory (Figure 6.4(a)-(b)).

Importantly, the *Heliconius erato* Learning group show a significantly higher total number of synapses in the calyx compared with the aged-matched Control group (Figure 6.5(c)). The experience of the Learning and Control groups was identical, save for the four-day “training period”, where for the Learning group specific colours were consistently presented with either a food reward or aversive stimulus, while for the Control group both colours were equally rewarded and punished. Both groups were then fed on only white feeders for the final eight days before dissection. In *Heliconius erato*, therefore, experience with a single, simple learning assay – a relatively modest environmental difference – is associated with maintaining higher numbers of synapses in the calyx. Increased synapse density or count in the mushroom body has previously been linked to visual [90] and olfactory [88,89] learning and long-term memory in Hymenoptera, and these results extend those findings to visual learning and memory in a *Heliconius* butterfly. Remarkably, these differences between the *Heliconius erato* Learning and Control groups persisted for eight days after their exposure to coloured feeders, suggesting that learning-induced synaptic connections in the calyx are being maintained in *Heliconius erato* for considerable amounts of time after exposure. To my knowledge this is the longest example of persistence of a learned association in an insect without reinforcement, besting a seven-day period in the ant *Camponotus blandus* [383]. Interestingly, the total synapse counts in the calyx were not significantly different between the *Heliconius erato* and *Dryas iulia* Control groups, potentially suggestive of rather aggressive synaptic pruning in the *Heliconius erato* Control individuals when kept in a relatively simple environment.

Despite learning the food-colour association, the *Dryas iulia* Learning group did not exhibit significant differences from the Control group, although the groups did exhibit a similar
trend to *Heliconius erato* (Figure 6.5(c)). As butterflies were sampled eight days after exposure to the Learned colour cues, one possibility is that the Learning *Dryas iulia* did indeed initially exhibit a higher calyx synapse count after the training period, which was then reduced while spending eight days on white feeders, whereas *Heliconius erato* maintained the synapses over that period. Alternatively, *Dryas iulia* may simply never have experienced a learning-induced increase in calyx synapses. The sampling timeframe of this experiment means it is impossible to distinguish between these possibilities with the present dataset. Nevertheless, the difference in synapse number between Learning and Control *Heliconius erato*, and the absence of group differences in *Dryas iulia*, is reflected in the markedly superior performance of *Heliconius erato* in the eight-day recall test (Figure 6.4(a)-(b)). This suggests that the improved long-term memory of *Heliconius erato* is, at least partly, facilitated by an increased synaptic flexibility in the calyx of the mushroom body. A similar interspecific difference in the plasticity of a particular brain region has been found in birds. In the food-storing marsh tit hippocampus volume was observed to increase in response to food-storing experience, but the non-storing blue tit exhibited no such change [75].

Interestingly, neither *Heliconius erato* nor *Dryas iulia* showed a learning-associated increase in calyx volume. Rather, learning and memory formation in *Heliconius erato* appears solely achieved through synaptic reorganisation within a neuropil of constant volume. This mirrors findings in honeybees [88] and leaf-cutting ants [89], where olfactory learning was linked to increased synapse density without expansion of the calyx. In contrast, learning-induced calyx growth has been linked to visual learning in honeybees [90] and host plant learning in the butterfly *Pieris rapae* [216]. While *Heliconius erato* did exhibit growth in the calyx with age, but not learning, this was achieved with a relatively constant number of Kenyon cells (Figure 6.5(b),(d), Figure 6.6(b)). While there were no intraspecific differences in Kenyon cell counts (Figure 6.5(d), Figure 6.6), I did detect evidence of slight differences in Kenyon cell number when pooling species and age-matched groups. However, the effect was minor and cannot explain the dramatic posteclosion expansion of the calyx in *Heliconius erato*. Calyx growth in *Heliconius erato*, therefore, seems primarily driven by increased dendritic growth, rather than neurogenesis (Figure 6.6(b)). Furthermore, this study is the first to measure variation in both Kenyon cell and synapse number in response to learning experience, allowing me to investigate how their relationship varies. Notably, the increased number of synapses in the calyces of the *Heliconius erato* Learning group was not mirrored by a corresponding increase in Kenyon cell number (Figure 6.5(d)). Instead, learning was associated with an
increase in the number of synapses per Kenyon cell (Figure 6.6(a)). Kenyon cell neurogenesis has been detected in other insects [353,355,356], but is notably lacking in honeybees [357]. Together with the present findings on *Heliconius erato*, this suggests that, in insects at least, sophisticated cognitive ability can be achieved without neurogenesis, despite its importance in vertebrates [346,348–352]. Interestingly, the relationship between the number of Kenyon cells and synapses does not scale isometrically between *Dryas iulia* and *Heliconius erato* (Figure 6.6(a)). Instead, the larger mushroom bodies of *Heliconius erato* have fewer synapses per Kenyon cell. This suggests that an increased synapse count requires a proportionately higher increase in Kenyon cell number.

I also investigated potential relationships between neuroanatomical traits in the mushroom body and individual performance in the initial recall and long-term memory tests (Figures 6.7–6.9). In *Heliconius erato* performance in the initial recall test (16 hours after final exposure to the learning environment) was positively correlated with synapse density and total number, and with the ratio of synapses to Kenyon cells (Figure 6.7(g),(i),(k)). Although one study in honeybees found no relationship between synapse density in the calyx and performance in a challenging foraging task [367], these results for *Heliconius erato* are consistent with the findings in another honeybee study which reported a similarly positive correlation between synapse density in the mushroom body collar, an area of the calyx receiving solely visual input, and recall accuracy [90]. However, one caveat attached to this result is that, without further experiments, it is not possible to determine the direction of causality – whether individuals with more calyx synapses had better recall or, rather, whether individuals that learned the association more strongly had a corresponding increase in synapses. In contrast with *Heliconius erato*, although *Dryas iulia* did learn the colour association (Figure 6.4(a)), they did not show a similar relationship between recall performance and synapse density or count (Figure 7). This is suggestive of the mushroom bodies in *Dryas iulia* playing a diminished role in consolidating the learned food-colour association. *Dryas iulia* did, however, exhibit a surprising negative correlation between calyx volume and initial recall accuracy (Figure 6.7(b)).

Unlike for the initial recall test, neither species showed any correlation between performance in the long-term recall test and measured neuroanatomical traits (Figure 6.8). This is particularly surprising given that butterflies were dissected only hours after completing this trial, but eight days after the initial recall test. However, for *Heliconius erato*, this result did seem to be disproportionately affected by the presence of a single outlier individual. This
individual was very accurate during the first recall test, but then showed very poor recall in the long-term memory trial (Figure 6.4(b)), despite having a high synapse count (Figure 6.7(i),(k)). When this individual was removed from analysis, the relationship between long-term recall performance in *Heliconius erato* was positively associated with synapse number and the ratio of synapses to Kenyon cells, consistent with the effect seen for initial recall performance. Taken together with the results for the initial recall test, this is highly suggestive of investment in increased synaptic connections in the mushroom body playing a key role in long-term memory consolidation.

In conclusion, this study presents the first measurements of both cognitive performance and several neuroanatomical traits in the mushroom body in a comparative framework across two closely related insect species. These results, thus, provide crucial insight into the mechanisms underpinning variation in cognitive performance both within and between species. The present findings show a dramatic difference between *Heliconius erato* and *Dryas iulia* in the effects of aging and learning experience on the mushroom body. Overall, the mushroom bodies of *Heliconius erato* showed far greater plasticity, exhibiting a response to learning experience specifically, in the form of increased synapse number in the calyx. This suggests that the enhanced learning ability of *Heliconius erato* is supported, not just by an increase in mushroom body size (Chapter 2), but also by increased synaptic flexibility in response to learning experience. This increased neural plasticity in the mushroom body appears primarily underpinned by synaptic reorganisation and not neurogenesis, indicative of a potentially evolutionary convergence with honeybees [357]. Crucially, performance in the initial recall test was positively correlated with synapse count and density, and synapses per Kenyon cell. Microglomeruli in the calyx are formed from synaptic connections between Kenyon cells and projection neurons from the optic and antennal lobes. These results suggest that learning and memory formation is facilitated by increased connectivity between these neurons. This adds to a growing body of evidence implicating the microglomeruli structure of the mushroom body in learning and memory consolidation [88,89,367]. The present findings could be expanded by conducting the same experiment on a wider sample of Heliconiini to determine whether the differences here detected between *Heliconius erato* and *Dryas iulia* can be generalised to a general difference between *Heliconius* and non-*Heliconius* Heliconiini. Nevertheless, by combining behavioural data with measurements of several large- and fine-scale neuroanatomical traits across two species, this study is a crucial step towards a detailed understanding of interspecific variation in cognitive ability.
7 Non-elemental learning in Heliconiini butterflies

7.1 Introduction

The ability to learn associations between environmental cues and resources is crucial for animals foraging in varied and changing environments. Many animals are capable of being classically conditioned to form such associations [384,385]. Typically, these involve learning to associate a single conditioned stimulus with a single unconditioned stimulus through unambiguous reinforcement [386,387], so-called “elemental” learning. However, while foraging in natural environments, an animal is likely to encounter complex stimuli comprised of several constituent elements, such as combinations of colours and odours, or spatial cues. Through elemental learning, a compound stimuli AB would be represented as the sum of A and B, which each become associated with the unconditioned stimulus [386]. Ambiguity can thus arise where compounds have shared elements. For example, a negative patterning task requires discriminating between reinforced elements A and B, and their non-reinforced combination AB (A+, B+ vs AB-). This task is impossible to solve through elemental learning, but requires treating AB, not as the sum of A and B, but as a distinct configuration, this ability is referred to as “non-elemental” learning [388,389].

Among arthropods, the spiny lobster Panulirus argus [390], honeybees and bumblebees [391–396], and, recently, Drosophila [397] are known to be capable of solving such non-elemental discrimination tasks. However, there is scant evidence on non-elemental learning in insects outside of this handful of well-studied species. There is also evidence to suggest that the mushroom bodies may play a key role in non-elemental learning for some groups. In honeybees, experimental inhibition of the mushroom bodies impaired the ability to solve non-elemental olfactory discrimination problems, but not elemental discriminations [102]. Additionally, computer modelling has suggested that the neuroarchitecture of the mushroom bodies is sufficient for solving non-elemental learning tasks [398]. However, it is unknown
how widespread this ability is in insects, how it varies across species and sensory modalities, and how this variation links to mushroom body size and structure.

The neotropical butterfly genus *Heliconius* shows an extraordinary expansion of the mushroom bodies, which are 3-4 times larger than in other Lepidoptera, even compared with closely Heliconiini-related genera (Chapter 2) [129,130]. The Heliconiini tribe emerged relatively recently, approximately 25 to 30 million years ago [131], providing an excellent comparative framework for investigating the functional consequences of mushroom body expansion on non-elemental learning across a small phylogenetic scale. Mushroom body expansion in *Heliconius* has been speculatively linked to their unique foraging behaviour as the only Lepidoptera known to actively feed on pollen [129,130,399]. Pollen is primarily collected from the pollen-rich cucurbitaceous vines *Psiguria* and *Gurania* [133,137–139]. *Heliconius* use this dependable, but scarce, resource by establishing ‘traplines’, stable foraging routes along which specific plants are repeatedly visited [133,142,143]. This suggests *Heliconius* possess a sophisticated capacity for spatial learning using learned visual landmarks, similar to behaviours observed in certain bees [201,202]. Such navigation, presumably depends, in part, on the ability to learn associations with complex, visual, configural stimuli. For example, in whip spiders navigation depends on the learning of configural cues [400]. In *Heliconius*, time may also form part of multimodal cues used in their traplining behaviour [133,277]. Importantly, although within Lepidoptera, elemental colour learning ability is widespread [217,220,339,401,402], non-elemental learning has not been demonstrated in any sensory modality.

Based on these neuroanatomical and ecological factors, I expect *Heliconius* to outperform other Heliconiini in configural learning tasks. To test this, I compared the performance of *Heliconius erato*, *Heliconius melpomene* and the closely-related Heliconiini *Dryas iulia*, which possess markedly smaller mushroom bodies than *Heliconius* (Chapter 2), in two learning tasks of differing complexity – positive patterning and biconditional discrimination. Positive patterning requires the subject to learn to discriminate between the rewarded combination AB and its non-rewarded, constituent elements A and B (A- and B- vs AB+). However, while positive patterning involves configural cues, it is possible to solve this task using an elemental strategy if the associative strength of each individual element is below a response threshold, but combined that threshold is exceeded. Positive patterning is, therefore, not strictly a test for non-elemental learning. Biconditional discrimination tasks, conversely, can only be solved through non-elemental learning [403]. Here, four elements (A, B, C, D) are
combined to form four distinct cues, two of which are reinforced and two not (AB+ and CD+ vs AC- and BD-). Every element is therefore represented equally by both outcomes and the task can only be solved by learning to discriminate between the compounds.

Mushroom body expansion in Heliconius is primarily driven by visual, rather than olfactory, inputs (Couto et al, in prep.). For these experiments, I therefore used visual cues comprised of combinations of different colours, partly based on a protocol demonstrating visual non-elemental learning in honeybees [391]. This contrasts with the majority of investigations into non-elemental learning insects, which have mostly used mixtures of olfactory cues [102,390,392,393,395,397], with visual non-elemental discrimination only demonstrated in honeybees and bumblebees [391,394,396]. This experiment offers the first test of non-elemental learning within Lepidoptera, and the first study of visual non-elemental learning in an insect outside of Hymenoptera. I hypothesise that the expanded mushroom of Heliconius will facilitate an enhanced non-elemental learning ability relative to Dryas iulia.

7.2 Methods

i) Animal husbandry and pre-training

Both the positive patterning and biconditional discrimination assays were conducted using Dryas iulia and Heliconius erato, with the positive patterning assay also including Heliconius melpomene. All experiments were conducted using freshly-eclosed individuals only.

The positive patterning experiment was conducted in at the Smithsonian Tropical Research Institute insectaries in Gamboa, Panama. For the positive patterning assay, stock populations of Dryas iulia, H. erato and H. melpomene were established from wild-caught individuals around Gamboa, Panama. Stock populations were kept in outdoor cages in ambient conditions. Adult stock butterflies were fed daily with a sugar-protein solution (20% sugar, 5% Vertark Critical Care Formula, 75% water, w/v) mixture and had access to Psiguria and Lantana flowers. Larvae of both species were fed with Passiflora biflora leaves. The positive patterning assay was conducted using only captive-reared butterflies from these stock populations.

The biconditional discrimination trials were conducted in greenhouses at the University of Bristol. For this assay, Dryas iulia and H. erato pupae were shipped to Bristol from the Stratford Butterfly Farm (UK) and Costa Rica Entomological Supply. Pupae were kept in a
climate-controlled greenhouse at 30°C and 80% humidity until emergence. The biconditional
discrimination assay was conducted using adults that emerged from these pupae.

For both experiments, the day after eclosion experimental individuals were introduced
to a pre-training cage containing only white feeders filled with sugar-protein solution. Individuals were kept in this pre-training environment for two full days to acclimatise to using the feeders before beginning training.

**ii) Positive patterning assay**

After pre-training, butterflies were subject to an initial preference test between three types of artificial feeders: yellow, purple, yellow + purple. Butterflies were introduced to a testing cage containing 12 artificial feeders (4 yellow, 4 purple and 4 yellow + purple) arranged randomly with at least 6.5 cm between feeders on each side. To ensure that butterflies responded to visual cues only, feeders in the testing cages were empty. Butterflies were deprived of food from 12:00 the day prior to testing to encourage feeding during the trials. The preference test lasted for four hours and was filmed using mounted GoPro Hero 5 cameras. The film was then reviewed to count the number of feeding attempts per individual on each colour. A feeding attempt was only counted if the butterfly landed on the feeder and probed it with its proboscis.

After the initial preference test, butterflies were placed in training cages containing 12 feeders (4 yellow, 4 purple and 4 yellow + purple). The yellow + purple feeders contained a food reward while the yellow and purple feeders were filled with a saturated solution of quinine, serving as an aversive stimulus. These feeders were arranged randomly each morning. Butterflies kept in training cages for eight days and could freely sample the feeders for the entire period. Following the training period, butterfly feeding preferences were re-tested, following the same protocol as the initial preference test.

**iii) Biconditional discrimination assay**

The biconditional discrimination assay used artificial feeders of four different colour combinations: red + blue, purple + yellow, red + yellow, purple + blue (Figure 7.1). Following the pre-training period, butterflies were placed in a cage containing four empty feeders of each colour combination to test for their initial preference, following the testing protocol described for the positive patterning assay. Butterflies were then randomly assigned to one of two training regimes.
For the first training regime, butterflies were introduced to a cage containing four feeders of each colour combination, with the purple + yellow and blue + red feeders containing a food reward and the yellow + red and blue + purple combination filled with the aversive quinine solution (Figure 7.1). Each colour was therefore evenly represented between rewarded and punished feeders. Accordingly, butterflies could not solve the task by learning a single colour but needed to learn specific combinations of colours. The training regime for the second group was the reverse of the first, with yellow + red and purple + blue feeders rewarded and purple + yellow and blue + red punished. The eight-day training period and subsequent testing followed the same protocol as the positive patterning assay.

Figure 7.1 The four colour combinations used during the biconditional discrimination trial. These pairs of combinations were presented with either a food reward (sugar-protein) or aversive stimulus (quinine). Each colour was represented in two different combinations.

iV) Statistical analyses

Learning performance in the positive patterning and biconditional discrimination assays was analysed with generalised linear mixed models (GLMMs) using the glmer function from the R package lme4 v 1.1-27.1 [293]. These models included species and training as fixed effects (in addition to their interaction), with an individual-level random effect. For the biconditional discrimination assay, since different butterflies were trained to different colour combinations, training regime was also included as a fixed effect. However, training regime was non-significant and therefore removed from the model. Diagnostics for these models were assessed using the package DHARMa [294]. All post hoc comparisons were made by obtaining the estimated marginal means using the R package emmeans v 1.7.0 and were corrected for selected multiple comparisons using the Tukey test [315].
### 7.3 Results

#### i) Heliconius outperform Dryas iulia in a visual positive patterning task

Both training ($\chi^2=350.48$, d.f.=1, $P<0.001$) and species ($\chi^2=23.556$, d.f.=2, $P<0.001$) had a significant effect on the proportion of correct choices made (Figure 7.2). All species learned to associate the food reward with purple + yellow feeders (Figure 7.2, Table 7.1). However, after training, both *H. erato* and *H. melpomene* visited the rewarded purple + yellow feeders significantly more frequently than *Dryas iulia* (Figure 7.2, Table 7.1), indicating an ability to more accurately learn the combine purple and yellow cue ($\chi^2=71.342$, d.f.=2, $P<0.001$). Prior to training, there were no differences between species in their proportion of feeding attempts on the purple + yellow feeders (Figure 7.2, Table 7.1).

![Positive patterning learning in Heliconiini](image)

**Figure 7.2** Positive patterning learning in Heliconiini. Proportion of feeding attempts made on each colour combination before and after training for *Dryas iulia*, *H. erato* and *H. melpomene* before and after training. All three species learned to associate food with the purple + yellow combination feeders. However, *H. erato* and *H. melpomene* were far more accurate in learning this association (Table 7.1).
Table 7.1 Positive patterning learning in Heliconiini. Post-hoc comparisons, corrected for multiple comparisons using the Tukey test, between the proportion of correct feeding attempts by *Dryas iulia* (n=34), *H. erato* (n=26) and *H. melpomene* (n=13) before and after training.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Estimate</th>
<th>SE</th>
<th>Z ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dryas iulia</em> naive – <em>Dryas iulia</em> trained</td>
<td>-1.075</td>
<td>0.117</td>
<td>-9.205</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>H. erato</em> naive – <em>H. erato</em> trained</td>
<td>-2.611</td>
<td>0.162</td>
<td>-16.084</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>H. melpomene</em> naive – <em>H. melpomene</em> trained</td>
<td>-2.643</td>
<td>0.282</td>
<td>-9.370</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>Dryas iulia</em> naive – <em>H. erato</em> naive</td>
<td>0.184</td>
<td>0.201</td>
<td>0.914</td>
<td>0.943</td>
</tr>
<tr>
<td><em>Dryas iulia</em> naive – <em>H. melpomene</em> naive</td>
<td>0.585</td>
<td>0.298</td>
<td>1.961</td>
<td>0.369</td>
</tr>
<tr>
<td><em>H. erato</em> naive – <em>H. melpomene</em> naive</td>
<td>0.401</td>
<td>0.313</td>
<td>1.281</td>
<td>0.796</td>
</tr>
<tr>
<td><em>Dryas iulia</em> trained – <em>H. erato</em> trained</td>
<td>-1.353</td>
<td>0.176</td>
<td>-7.673</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td><em>Dryas iulia</em> trained – <em>H. melpomene</em> trained</td>
<td>-0.983</td>
<td>0.232</td>
<td>-4.242</td>
<td>0.0003***</td>
</tr>
<tr>
<td><em>H. erato</em> trained – <em>H. melpomene</em> trained</td>
<td>0.370</td>
<td>0.245</td>
<td>1.511</td>
<td>0.657</td>
</tr>
</tbody>
</table>

**ii) Heliconius erato, but not Dryas iulia, solves a visual biconditional discrimination task**

*Heliconius erato* and *Dryas iulia* differed in their response to biconditional discrimination training ($\chi^2$=20.727, d.f.=1, $P<0.001$). *Heliconius erato* significantly shifted their feeding attempts towards the trained combinations, indicating a non-elemental learning of the colour combinations (Figure 7.3, Table 7.2). Conversely, *Dryas iulia* showed no evidence of learning in the biconditional discrimination task (Figure 7.3, Table 7.2). There was no significance difference between species in feeding preference prior to training (Z ratio=-0.920, $P=0.794$).

![Figure 7.3](image-url)
Table 7.2 Biconditional discrimination in Heliconiini butterflies. Post-hoc comparisons, corrected for multiple comparisons using the Tukey test, between the proportion of correct feeding attempts by Dryas iulia (n=14) and H. erato (n=13) before and after training.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Estimate</th>
<th>SE</th>
<th>Z ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dryas iulia naive – Dryas iulia trained</td>
<td>-0.2384</td>
<td>0.192</td>
<td>-1.241</td>
<td>0.6005</td>
</tr>
<tr>
<td>H. erato naive – H. erato trained</td>
<td>-1.5566</td>
<td>0.217</td>
<td>-5.465</td>
<td>&lt;.0001***</td>
</tr>
</tbody>
</table>

7.4 Discussion

I compared the ability of Heliconius erato, Heliconius melpomene and the closely-related Dryas iulia to solve complex, visual associative-learning tasks. All species were tested in a positive patterning task, while Heliconius erato and Dryas iulia were further compared for performance in an unambiguously non-elemental, biconditional discrimination task. Based on their elaborated mushroom bodies (Chapter 2) [102], I predicted that Heliconius would show a superior non-elemental learning ability. Indeed, Heliconius outperformed Dryas iulia in both tasks (Figures 7.1 & 7.2). While all species were able to solve the positive patterning task, Heliconius were significantly more accurate (Figure 7.2). The biconditional discrimination task, however, was solved by Heliconius erato, but not by Dryas iulia (Figure 7.3).

The biconditional discrimination task could only be solved non-elementally by learning to discriminate between the combinations of colours [403], as every colour cue is represented equally by both a positive and negative outcome. The results of the biconditional discrimination trials unambiguously show that Heliconius erato are capable of non-elemental learning, providing the first evidence of this ability within Lepidoptera. Furthermore, within insects, visual non-elemental learning has only previously been demonstrated in honeybees and bumblebees [391,394]. In contrast, Dryas iulia showed no ability to solve the biconditional discrimination task. The present result is consistent with expansion of the mushroom bodies facilitating improved non-elemental learning ability in Heliconius. This finding also complements a study in honeybees showing that experimental blocking of the mushroom bodies impaired non-elemental, but not elemental, learning [102].

Dryas iulia were able to solve the positive patterning task but did so significantly less accurately than H. erato and H. melpomene. However, based solely on these results, it cannot be concluded that Dryas iulia are capable of visual non-elemental learning. Positive patterning
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tasks can potentially be solved through elemental learning if the associative strength of each element is below a response threshold which is exceeded when those elements are combined [392,404]. Nevertheless, honeybees appear to solve positive patterning tasks using non-elemental processing as blocking of the mushroom bodies impaired their ability to learn positive patterning, but not elemental discriminations [102]. In the present experiment, while I cannot determine the extent to which butterflies used elemental or non-elemental processing in the positive patterning assay, the significantly reduced accuracy of *Dryas iulia* relative to *H. erato* and *H. melpomene* is suggestive of the expanded mushroom bodies of *Heliconius* facilitating an enhanced ability to learn positive patterning, likely involving configural processing of the cues.

There are some potential criticisms of this experimental protocol that should be addressed. Firstly, it could be argued that non-elemental learning is not really being demonstrated here if the colour combinations were learned as a whole, rather than as composed of distinct elements. In this way, it follows, a biconditional discrimination task could be solved elementally, treating combinations, rather than colours, as elements. However, I would argue that learning the combination as a whole (separate from the sum of its colours), is in fact an example of configural learning. It is this process of treating the combination as a separate entity that represents a configural approach [404]. Indeed, the ability to solve either negative patterning or biconditional discrimination tasks based on colour combinations, has previously been taken as evidence of non-elemental, or configural, learning in honeybees [391,394] and bumble bees [396]. Secondly, it could be suggested that the difference in performance between *Heliconius erato* and *Dryas iulia* in the biconditional discrimination task could simple be explained by differences in the ability to visually discriminate between the colour combinations. This is possible, but the results of the positive patterning assay show that *Dryas iulia* is capable of recognising colour combinations (Figure 7.2). Moreover, the feeder presented were relatively large (far larger than *Lantana* flowers upon which wild *Dryas iulia* regularly feed upon) making it unlikely that they were not able to perceive the individual colour components of the flowers. It does not, therefore, seem likely that differences in perception alone explain these results.

The enhanced non-elemental learning ability of *Heliconius* compared with *Dryas iulia*, may have been driven by its pollen feeding behaviour, which relies on both sophisticated spatial navigation [133,142,143], and the ability to identify and learn relatively rare pollen sources - predominantly Cucurbitaceous vines [133,137–139]. Configural learning, particularly the
integration of multisensory cues, plays a crucial role in navigation for several arthropods including the wood ant *Formica rufa* [99] and the whip spider *Phrynus marginemaculatus* [400]. *Heliconius*' improved ability to learn more complex associative cues may have been driven by the cognitive demands of foraging for pollen. Like *Heliconius*, honeybees, which are similarly capable of solving a visual biconditional discrimination task [391], possess greatly expanded mushroom bodies with significant visual input [405] and rely heavily on visual cues during foraging flights [287,406–409]. The visual non-elemental learning abilities of *Heliconius* and honeybees, and elaborated mushroom bodies, may, therefore, represent an evolutionary convergence in response to similar selective pressures related to foraging strategy.

The present study can be expanded in several ways. Firstly, the performance of *Dryas iulia* and *H. erato* could be compared in a negative patterning trial (A+, B+ vs AB-). Like the biconditional discrimination task, non-elemental learning is necessary to solve negative patterning problems. However, it is arguably a cognitively easier task, involving only two colours across three combinations, rather than four colours in four different combinations, and could potentially be solved by *Dryas iulia*. Amongst insects, the ability to solve a negative patterning task has only been demonstrated in honeybees, bumblebees and *Drosophila* [391,392,394,397]. Secondly, although *Heliconius* outperformed *Dryas iulia* in configural learning tasks, it is uncertain whether a similar advantage would be seen using olfactory cues, as the mushroom body expansion of *Heliconius* is primarily driven by visual inputs (Couto et al. *in prep.*). Revealingly, the bumblebee is capable of visual non-elemental learning [394], but failed to solve an olfactory negative patterning task in a separate experiment [410]. Although these two bumblebee experiments are not directly comparable, it does suggest that visual and olfactory non-elemental learning ability can vary markedly within a single species.

In conclusion, this study provides the first evidence of the ability to solve a biconditional discrimination task among the Lepidoptera. Previously only demonstrated in the honeybee within the insects, the ability of *H. erato* to solve this relatively sophisticated cognitive problem highlights *Heliconius* as an important system for investigating higher order learning in insects. The corresponding inability of *Dryas iulia* to solve the biconditional discrimination task, and its poorer performance in the positive patterning trial, suggests that the expansion of the mushroom bodies in *Heliconius* may have facilitated an enhanced capacity for non-elemental learning.
8 Conclusions

8.1 Integrating comparative data on neuroanatomy and cognitive ability

The aim of this work was to investigate the ecological and evolutionary processes leading to interspecific variation in neuroanatomy and cognition, using Heliconiini butterflies as a novel system. Mushroom body function has been well studied within single species and across broad phylogenetic scales [71,83,86]. However, directly comparative interspecific studies of the mushroom body have been limited, particularly regarding the incorporation of behavioural data. This thesis presents the first comparative study of the mushroom bodies that combines data on cognition with both large- and fine-scale neuroanatomical measurements across several closely related species. Previous data from two Heliconius species and two closely-related Heliconiini had suggested that the Heliconius genus exhibits a dramatic expansion of the mushroom bodies [129,130]. Importantly, mushroom body expansion in Heliconius appears to have occurred relatively recently, with Heliconius diverging from their sister genus Eueides approximately 18 million years ago [131]. This contrasts with Hymenoptera, one of major insect groups for investigating the mushroom bodies, where a similar expansion appears to have occurred approximately 150 million years ago [21]. Such large phylogenetic distances present difficulty in conducting meaningful comparative behavioural experiments that test specific functional and adaptive hypotheses. Importantly, the neural elaboration in Heliconius co-occurs with the novel foraging strategy of traplining for pollen, which appears to depend on enhanced spatial learning and memory [132]. This provides a specific adaptive hypothesis that can be tested through comparative assays of cognition. Heliconius thus present a strong framework for the investigation of links between specific behaviours, their neural underpinnings and the selective pressures driving their evolution. With this thesis I hope to establish Heliconius as an excellent, new model for investigating how brain evolution is shaped by ecological factors.

In Chapter 2, I present phylogenetic comparative analyses showing that the entire Heliconius genus does, indeed, exhibit marked expansion of the mushroom bodies, relative to
all other Heliconiini (Figure 2.1). This expansion also appears associated with an increased evolutionary rate of mushroom body size at the base of the genus Heliconius (Figures 2.4 & 2.5), suggestive of an increased intensity of natural selection on this brain region. Importantly, neither the rest of the central brain, or major components of sensory systems that project to the mushroom bodies, the medulla of the optic lobe and the antennal lobe, show a similar expansion within Heliconius. Elaboration of the mushroom bodies appears to have occurred independently of other brain regions, placing Heliconius butterflies as an important example of mosaic evolution in the brain [20,36]. Such mosaic patterns of brain evolution previously identified include expansion of the neocortex in primates [20], and the hippocampus in food-storing birds [17,72–76]. In birds, hippocampus expansion facilitates enhanced spatial memory [77–82], potentially showing a particular convergence with mushroom body expansion in Heliconius.

Contrary to expectations, I found that within the Heliconiini mushroom body expansion did not occur just once along the branch leading to Heliconius. Rather, it also occurred independently in at least two further branches, at the base of Heliconius and Eueides, and in Dryadula phaetusa (Figure 2.1). This suggests that pollen feeding alone cannot explain all incidences of mushroom body expansion within the Heliconiini. One possibility is that certain behaviours prerequisite for pollen feeding, such as a stable-home range and roost-site fidelity, may have evolved along the branch leading to Eueides and Heliconius, driving an initial expansion of the mushroom body. This could later have been followed by a further, even more dramatic, expansion within Heliconius, itself driven by the cognitive demands of trapline foraging. However, the paucity of data on the extent to which other Heliconiini establish and maintain home ranges currently prevents the direct testing of this hypothesis. Although, interestingly, territorial behaviour has been observed in Eueides tales and Eueides aliphera [276]. Nevertheless, the distinct expansion of the mushroom bodies within Heliconius is consistent with a role of pollen feeding in driving this neural elaboration.

One difficulty in determining the selective pressures driving mushroom body expansion in Heliconius is its likely singular origin at the base of the genus (Figure 2.6(b)-(d)). This presents a difficulty in disentangling the general genus effect from a potential role of pollen feeding through the phylogenetic analyses of neural traits alone. Consequently, to further test this hypothesis I ran a series of comparative cognitive experiments across Heliconius and out-group Heliconiini species testing specific cognitive abilities presumed to be involved in
pollen feeding. However, to test this hypothesis more robustly I first investigated the role of an alternative ecological factor in Heliconius brain evolution – host plant use.

Heliconiini partially rely on leaf shape to visually identify and learn host plants [214], and this has been speculatively linked to mushroom body expansion [213]. However, in Chapter 4, I show that shape-learning ability is generally inconsistent across the Heliconiini. Importantly, that Heliconius are not superior shape learners than out-group species (Figure 4.5). In addition, I did not detect any relationship across the Heliconiini between relative mushroom body size and either host plant number or their leaf shape diversity (Figure 4.6). Together, these results suggest that the visual identification of host plants based on leaf shape has not been an important factor driving mushroom body expansion in Heliconius. This ostensibly leaves pollen feeding as the most plausible ecological factor driving Heliconius brain evolution.

In Heliconius, pollen feeding is a foraging strategy that seemingly relies on an advanced visual, spatial learning ability over large areas of up to 1 km² [133,142,143,292]. In Chapter 3, I tested the spatial learning abilities of Heliconius melpomene, showing that they can solve a large-scale T-maze over distances of approximately 70 metres. Unfortunately, due to high mortality rates I was unable to also test Dryas iulia and include a comparative element to the experiment. Nevertheless, while maze solving ability has previously been shown in other insects including honeybee Apis mellifera [287] and the ant Cataglyphis niger [288], this is the first demonstration of maze learning in Lepidoptera. Together with a recent study where experimentally displaced Heliconius will return to their original locations [205], this result shows that Heliconius are capable of learning spatial information on a relatively large scale, similar to abilities reported for orchid bees and bumblebees [125,126]. The ability to learn such spatial information seems a prerequisite for efficient traplining for pollen from geographically sparse sources [133], and is consistent with observations of wild Heliconius foraging with a high degree of spatial fidelity [133,142,143,292]. However, their mechanisms of navigation remain unknown. Field data suggests that Heliconius rely on learned visual landmarks [143,197,203], but this remains untested experimentally. Such an experiment could conceivably be conducted on wild communities of butterflies using artificially placed landmarks.

The considerable logistical difficulties associated with experimentally assessing spatial learning in Heliconiiini prevented me from expanding the experiment in Chapter 3 to include
more species, or mazes of greater complexity. However, traplining for pollen seemingly depends on several distinct cognitive abilities that are more tractable for testing in an interspecific comparative framework, namely, visual long-term memory, reversal learning and non-elemental learning (Chapters 5 & 7). These experiments showed *Heliconius* outperform closely-related Heliconiini in a visual long-term memory task (Figure 5.4(a)) and in non-elemental learning tasks (Figure 7.1, Figure 7.2). In fact, the accurate recall of a learned colour association after eight days removed from the cues is, to my knowledge, the longest demonstration of long-term memory in an insect. Given that neural expansion in *Heliconius* appears restricted to the mushroom bodies (Chapter 2), these results are highly suggestive of mushroom body elaboration playing a key role in facilitating these enhanced cognitive abilities.

Crucially, in Chapter 6, I present evidence that the mushroom body is directly involved in the formation of visual, long-term associative memories in *Heliconius erato* (Figures 6.5–6.7). This is the first experiment directly linking variation in cognitive ability between two closely related insects to variation in the mushroom body, providing a lucid example of how behavioural evolution can be facilitated by changes in the brain.

Through this combination of behavioural and neuroanatomical data, it seems clear that mushroom body expansion in *Heliconius* was, at least in part, driven by selection for enhanced visual learning and long-term memory. This is consistent with pollen feeding, which appears to depend on visually-learned cues [133,142,143], being a key evolutionary driver of this increase in mushroom body size. Indeed, *Heliconius* show an evolutionary convergence with apocritan Hymenoptera [405,411] and cockroaches [412,413], which have both evolved significant visual input to the mushroom bodies. In these groups, the mushroom bodies are essential for visually-oriented spatial learning and memory [21,97–99]. In Hymenoptera, mushroom body enlargement coincided with the evolution of parasitoidism, where host finding depends on spatial memory [21], while cockroach foraging centres on a stable shelter and employs learned visual spatial cues [414,414,415]. These behaviours show similarity with the roost-site fidelity and spatially consistent foraging routes of *Heliconius* [133,143,292]. Although I have not linked the *Heliconius* mushroom bodies to spatial memory specifically, such a role is highly plausible. The separate incidences of mushroom body enlargement in *Heliconius* and other insect groups, also show a potential convergence with the independent expansions of the hippocampus in a variety of food-storing birds [72,73,76,344,416], which has been linked to enhanced spatial memory [78–82]. Like the mushroom body (Chapter 2 [21]), the hippocampus shows evidence of mosaic evolution across birds [17,72–75] and
represents a similar instance of selection for increased volume in a specific region of the brain, likely driven by the cognitive demands associated with foraging strategy.

Interestingly, my behavioural experiments also showed that the enhanced cognitive abilities of *Heliconius*, relative to other Heliconiini, were limited to specific tasks, rather than an overall increase in “general intelligence”. Notably, *Heliconius* showed similar performance to out-group Heliconiini in reversal (Figure 5.4(b)) and shape (Figure 4.5) learning tasks. In the reversal learning task, all species showed a strong ability to learned reversed colour associations over a four-day training period (Chapter 4). It is possible that a more difficult reversal learning task may have revealed cognitive differences between *Heliconius* and other Heliconiini. However, similar tasks are routinely used as cognitive assays in other taxa, including vertebrates [332,334–338]. In the shape learning experiment, several *Heliconius* and non-*Heliconius* species successfully learned the associations, while *Dryas iulia* and *Heliconius hecale* showed no evidence of learning (Figure 4.5). This result indicates that mushroom body expansion in *Heliconius* was driven by selection for specific cognitive abilities, which must be understood with reference to the particular ecological challenges faced by the genus. A similar finding was made in two species of *Acanthodactylus* lizard, which differed in reversal learning performance, but not spatial memory [338]. This variation in relative cognitive performance between different tasks highlights the importance of conducting comparative cognitive experiments testing ecologically-relevant abilities [39,126]. Comparative behavioural experiments should be designed to test adaptive hypotheses informed by the cognitive ecologies of the animals involved. Importantly, poor performance in a given task should not necessarily be taken as evidence of overall cognitive ability [4,40–42]. Brain evolution is best be viewed as being shaped by selection for specific, ecologically-relevant behaviours, underpinned by neuroanatomy.

In Chapter 6, I provide evidence that that cognitive differences between *Heliconius erato* and *Dryas iulia* are facilitated not just by increased mushroom body size, but also increased synaptic plasticity in response to learning experience (Figures 7.5 & 7.6). Previous studies have found an association between synapose density in the calyx and learning within honeybees [88,90]. Additionally, interspecific variation in experience-related volumetric expansion of the calyx has been observed in *Polygonia* and *Aglais* butterflies [215]. However, to my knowledge, this is the first study directly connecting interspecific variation in mushroom body synaptic plasticity with variation in cognition. I also found that within *Heliconius erato*, but not *Dryas iulia*, synapse count in the calyx was positively correlated with individual
performance in a recall test. Very few studies have tested these links between individual cognitive ability and neuroanatomical measures at the synaptic level in insects. However, this result is consistent with a similar finding in honeybees which reported a positive association between synaptic density in the mushroom body collar, and learning speed and recall accuracy [90]. Together, these studies suggest that synaptic formation in the mushroom body calyx is an important neural correlate of learning performance, but it is crucial to test these links in a wider range of species. The findings in Chapter 6 highlight that cognitive evolution is associated with not just variation in the size of specific brain regions, but also their underlying connectivity and response to experience, even amongst closely related species [417]. Interestingly, in *Heliconius* this learning-induced increase in synapse density is achieved without any major neurogenesis of Kenyon cells (Figure 5.6). Instead, increased synapse counts appear to be a result of increased connectivity between existing Kenyon cells and projection neurons from the antennal and/or optic lobes within the calyx. This is consistent with an absence of neurogenesis in adult honeybees [418], but contrasts with the importance of adult hippocampal neurogenesis for spatial learning in birds and rodents, for example [351]. It seems then, that despite broad convergences in cognitive evolution, discussed above, there are also important differences, at the cellular and synaptic level, in the evolution of the mechanisms supporting learning and memory.

Overall, my work on the cognitive evolution of *Heliconius* butterflies comprises a synthesis of ecological, behavioural, and neuroanatomical data analysed within an interspecific, comparative framework. Together, these data reveal a dramatic expansion of the mushroom bodies within *Heliconius*, facilitating an improved visual long-term memory and non-elemental learning ability, potentially driven by the cognitive demands of trapline foraging for pollen. These findings, linking a change in size and underlying synaptic connectivity of a particular brain region to enhancement in specific, ecologically-important cognitive abilities, position *Heliconius* as an important case study in the evolution of brains and behaviour.

### 8.2 Reflections and the future of *Heliconius* as a model in cognitive evolution

As with any body of scientific research, there are some limitations to my data and analyses that warrant discussion. First is the fact that the emergence of pollen feeding, and the marked, *Heliconius*-specific expansion of the mushroom bodies both occurred along the branch leading to *Heliconius* (Chapter 2). Consequently, it is difficult to disentangle the effect of pollen
feeding from a general genus effect when investigating the selective pressures driving mushroom body expansion within *Heliconius*. Data on the non-pollen-feeding “Neruda” clade of four *Heliconius* species could potentially help to address this issue. One Neruda species, *Heliconius aede*, was included in the phylogenetic analysis presented in Chapter 1, showing mushroom bodies within the size range of other *Heliconius*, although towards the lower end. This indicates that, for at least this species, the loss of pollen feeding was not associated with a corresponding reduction in mushroom body size. However, this is far from providing a definitive answer to the question, particularly since there is no data on the wild foraging behaviour of the Neruda clade, including the presence or absence of traplining.

As revealed in Chapter 2, there were, in fact, several separate incidences of mushroom body expansion within the Heliconiini in addition to the dramatic enlargement in *Heliconius*. It is clear, therefore, that pollen feeding alone cannot account for all variation in mushroom body size across the Heliconiini. However, a lack of ecological data on the non-*Heliconius* members of the tribe, particularly relating to their foraging behaviours, currently prevents a proper investigation of these separate expansions. There is, therefore, a need to collect more data on the behaviours of these species in the wild, particularly relating to foraging strategy and roost-site fidelity. Furthermore, as described in Chapter 1, pollen feeding and mushroom body expansion are seemingly linked with a complex network of other traits, particularly relating to reproductive longevity and adult toxicity (Figure 1.3). It is thus difficult to disentangle the causal relationships in the evolution of these traits, which in some cases appear interdependent. Analysis of the interactions between these traits could potentially be approached using mathematical models that formalise their interdependencies [419] or agent-based simulations that reveal a hierarchy of competitive advantages provided by different traits [420]. This provides one route to reconstructing the order in which these traits changed, and their interdependencies, highlighting which were key to the expansion of the mushroom bodies.

Through my comparative behavioural experiments, I aimed to test specific adaptive hypotheses regarding mushroom body expansion, relating to both pollen feeding and host plant use. The most direct way to test whether this expansion relates to the cognitive demands of trapline foraging would be a comparative spatial learning experiment. I was able to test spatial learning in a single *Heliconius* species (Chapter 3). However, logistical difficulties associated with conducting that experiment at a facility in southern France prevented the inclusion of more species, including outgroup Heliconiini. One way to conduct such a comparative experiment would be in a large-scale arena located in a tropical environment where Heliconiini rearing is
feasible. However, currently no such facility currently exists. Such an experiment could also potentially benefit from the use of radar-tracking technology to record the flight paths of individual butterflies, which has previously been successfully demonstrated in several insects, including butterflies [421–426]. For such a strategy to work, it would be necessary to show that the tracking tag can be attached to the butterfly without causing physical harm, or significantly affecting natural flight behaviours. However, my own experience suggests currently available tags are likely too heavy for natural flight in Heliconius.

Chapters 5 and 6 together provide compelling evidence for a role of the mushroom bodies in the formation and maintenance of visual long-term memories in Heliconius. However, these results could further be supported by ablation experiments that block the function of the mushroom bodies [101]. This technique has previously been used to show the mushroom body plays an essential role for specific cognitive tasks in cockroaches [97], honeybees [102], Drosophila [95] and ants [98,99]. In addition, it is unclear whether Heliconius also possess an enhanced olfactory, and not just visual, long-term memory ability. Given that mushroom body expansion in Heliconius is primarily driven by increased visual, rather than olfactory, inputs to the calyx (Couto et al. in prep.), there is some reason to doubt this. Repeating the long-term memory experiment in Chapter 5 using olfactory rather than visual cues could reveal that Heliconius’ improved long-term memory is restricted to visual associations only, providing further links between neuroanatomical and behavioural evolution.

Like most comparative work, I was often faced with the challenge of balancing collecting appropriate sample sizes with including several different Heliconiini species – these two factors can often conflict with each other due to practical limitations of time and space. To generalise findings from the species tested to whole groups it is important that the species included in the experiment are phylogenetically representative. The shape learning, long-term memory, and reversal learning experiments presented in Chapters 4 and 5 were conducted across three Heliconius and three out-group Heliconiini species, comprising a phylogenetically broad sampling of the tribe. That diversity of species provides a sound foundation for interpolating my findings to all Heliconiini. Unfortunately, complications associated with the COVID-19 pandemic required me to reduce the scope of the non-elemental learning experiment to just two species, Heliconius erato and Dryas iulia. While the findings there are indicative of a potentially broad difference in non-elemental learning between Heliconius and other Heliconiini, those results could be corroborated by repeating the experiment with more species. Nevertheless, many interspecific comparative studies are conducted with a limited
number of species [75, 215, 338, 363]. Ideally, I would also have included *Eueides*, the sister genus of *Heliconius*, and non-pollen-feeding Neruda *Heliconius* in my behavioural experiments. Unfortunately, both groups present substantial difficulties in rearing, making them far less tractable than the species tested.

In Chapter 6, I shed light on the neural mechanisms behind the enhanced visual long-term memory of *Heliconius*. However, the potential role of differential gene expression in learning and memory formation within the Heliconiini is unstudied. Among insects, differential gene expression in the brain associated with learning or memory has been identified in bumblebees [427], *Drosophila* [428] and honeybees [429]. An investigation of learning- or memory-associated gene expression in the mushroom bodies of Heliconiini, could potentially offer important insight into the genetic underpinning of enhanced learning and memory in *Heliconius*. Importantly, the combination of RNA-seq [430] and ATAC-seq [431] offers a powerful way of assessing changes in DNA accessibility and gene expression [432]. A complementary approach is to assess the role of DNA methylation in learning and memory, which has been shown to play a significant role in memory formation in honeybees [433, 434], and is present in Lepidoptera [435]. Experiments blocking DNA methyltransferases in Heliconiini during an associative learning task, following methods previously demonstrated in honeybees [434], could be particularly illuminating.

Finally, elaboration of the mushroom bodies in *Heliconius* co-occurs with a dramatic increase in lifespan, which can extend up to six months, compared with four to six weeks in other Heliconiini [140]. This makes *Heliconius* a potentially fruitful system for investigating the mechanisms underpinning delayed cognitive senescence. The ability to maintain long-term memories of foraging routes suggests *Heliconius* may mitigate the age-related declines in cognition reported in other insects [436–439]. Uncovering the mechanisms by which *Heliconius* possibly delay cognitive senescence over an extended lifespan could provide valuable insight into aging processes. This topic is currently being investigated by Jessica Foley, a PhD student working in the Evolution of Brains and Behaviour Lab, who found that, interestingly, neither *Heliconius hecale* nor the closely-related Heliconiini *Dryas iulia* exhibited age-related declines in long-term memory of a colour cue (Foley et al., *unpubl. data*).

In conclusion, my findings provide a strong foundation for establishing *Heliconius* as a model for investigating the evolution of neural elaboration and behavioural sophistication, with several clear paths for expanding upon my work. Through the integration of life history,
behavioural, neuroanatomical, and genomic data, *Heliconius* is well placed to become a highly informative model system for researching cognitive evolution.
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