

1 **Title:**

2 Brain composition in *Heliconius* butterflies, post-eclosion growth and experience  
3 dependent neuropil plasticity

4

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24

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35 **ABSTRACT**

36

37 Behavioral and sensory adaptations are often based in the differential expansion of  
38 brain components. These volumetric differences represent differences in investment,  
39 processing capacity and/or connectivity, and can be used to investigate functional and  
40 evolutionary relationships between different brain regions. Here, we describe the  
41 brain composition of two species of *Heliconius* butterflies, a long-standing study  
42 system for investigating ecological adaptation and speciation. We confirm a previous  
43 report of striking mushroom body expansion, and explore patterns of post-eclosion  
44 growth and experience-dependent plasticity in neural development. This analysis  
45 uncovers two phases of post-emergence mushroom body growth comparable to those  
46 of foraging hymenoptera, but also identifies plasticity in several other neuropil. An  
47 interspecific analysis suggests *Heliconius* may display remarkable levels of  
48 investment in mushroom bodies for a Lepidopteran, and indeed rank highly compared  
49 to other insects. We also describe patterns of adaptive divergence in the volume of  
50 both peripheral and central neuropil within *Heliconius*, and across Lepidoptera, that  
51 suggest changes in brain composition plays an important role in ecological adaptation.  
52 Our analyses lay the foundation for future comparative and experimental analyses that  
53 will establish *Heliconius* as a useful case study in evolutionary neurobiology.

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## 68 INTRODUCTION

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70 Behavioral adaptations can allow populations to respond to environmental change and  
71 to invade new ecological niches. These behavioral changes are largely based in  
72 adaptive changes in brain function, which may in turn involve changes in the size and  
73 macro-structure of the brain (e.g., Gonda et al., 2009a,b, 2013; Park and Bell, 2010).  
74 For example, a clear signature of adaptive, phylogenetic divergence in brain  
75 composition in response to ecological variation is seen within many invertebrate and  
76 vertebrate species in their sensory neuropil (e.g. Barton et al., 1995; Huber et al.,  
77 1997; Gronenberg and Hölldobler, 1999; O'Donnell et al., 2013; Montgomery and  
78 Ott, 2015). In this case, nocturnal species, or those occupying low light environments,  
79 tend to have larger olfactory neuropil, and smaller visual neuropil (Barton et al., 1995;  
80 Montgomery and Ott, 2015).

81 Beyond the primary sensory regions, ecological selection pressures may have  
82 diverse effects on higher brain centers. The complexity of the physical (Capaldi et al.,  
83 1999; Safi and Dechmann, 2005; Pollen et al., 2007; Shumway, 2008; Farris and  
84 Schulmeister, 2011) or social environment (Barton and Dunbar, 1997; Burish et al.,  
85 2004; Lihoreau et al., 2012) have a detectable influence in shaping brain size and  
86 structure. For example, in vertebrates, this is clearly manifest in an evolutionary  
87 association between the need for spatial memory and the volume of the hippocampus  
88 (Clayton and Krebs, 1995; Garamszegi and Eens, 2004). In insects a similar  
89 association is found with the mushroom bodies (Farris and Schulmeister, 2011).

90 Differential expansions of individual brain structures shed light not only on  
91 species-specific biology and neuroecology, but more generally on the functional  
92 relationships between brain components (Barton and Harvey, 2000; Whiting and  
93 Barton, 2003), the relative strength of developmental constraints (Finlay and  
94 Darlington, 1995; Barton and Harvey, 2000), and on how very different brains  
95 produce seemingly similarly complex behavior (Giurfa and Menzel, 2001; Chittka  
96 and Niven, 2009; Farris, 2013).

97 Given their longstanding role in studies of both adaptation and brain function,  
98 the Lepidoptera are perhaps an underutilized group for integrating these fields to  
99 investigate evolutionary neurobiology. The Neotropical genus *Heliconius*  
100 (Heliconiinae, Nymphalidae) is well known for its diversity of bright warning patterns

101 that are often involved in Müllerian mimicry, where two or more unpalatable species  
102 converge on the same warning-signal to more efficiently advertise their  
103 distastefulness to predators (Müller, 1879; Mallet & Barton 1989; Merrill et al 2013).  
104 Perhaps as a result these butterflies have been intensively studied, leading to insights  
105 into a range of areas including population and community ecology, evolutionary  
106 genetics and development, as well as more generally contributing to our  
107 understanding of adaptation and speciation (Merrill et al., 2015).

108 *Heliconius* display a strong pattern of ecological divergence (Boggs et al.,  
109 1981; Estrada and Jiggins, 2002; Jiggins, 2008), and a number of striking behavioral  
110 adaptations (Gilbert, 1972, 1975; Mallet, 1986). Chief among these is a dietary  
111 adaptation, unique among Lepidoptera; adult pollen feeding (Gilbert, 1972, 1975).  
112 With the exception of four species formerly ascribed to the genus *Neruda* (Beltrán et  
113 al., 2007; Kozak et al., 2015), all *Heliconius* actively collect and ingest pollen as  
114 adults. This provides a rich source of amino acids and permits a greatly extended  
115 lifespan of up to six months without reproductive senescence, and shifts the energetic  
116 costs of chemical defense to larvae (Gilbert, 1972; Benson, 1972; Ehrlich and Gilbert,  
117 1973; Dunlap-Pianka et al., 1977; Cardoso and Gilbert, 2013). Without access to  
118 pollen *Heliconius* suffer a major reduction in longevity and reproductive success  
119 (Gilbert, 1972; Dunlap-Pianka et al., 1977; O'Brien et al., 2003).

120 Adult *Heliconius* collect pollen from a relatively restricted range of mostly  
121 Cucurbitaceous plants (Estrada and Jiggins, 2002), which are spatially dispersed and  
122 occur at low densities (Gilbert, 1975). Several lines of evidence suggest selection for  
123 pollen feeding has shaped *Heliconius* foraging behavior. Individuals inhabit home  
124 ranges of typically less than 1 km<sup>2</sup>, within which individuals repeatedly utilize a small  
125 number of roosting sites that they return to with high fidelity (Turner, 1971; Benson,  
126 1972; Gilbert, 1975; Mallet, 1986; Murawski and Gilbert, 1986; Finkbeiner, 2014).  
127 On leaving the roost individuals visit feeding sites with a level of consistency in time  
128 and space that strongly suggests 'trap-lining' behavior (Ehrlich and Gilbert, 1973;  
129 Gilbert, 1975, 1993; Mallet, 1986), analogous to that observed in foraging bees  
130 (Janzen, 1971; Heinrich, 1979). Roosts themselves are located visually (Jones, 1930;  
131 Gilbert, 1972; Ehrlich and Gilbert, 1973; Mallet, 1986), and older individuals tend to  
132 be more efficient foragers (Boggs et al., 1981; Gilbert, 1993). Together these  
133 observations suggest the evolution of pollen feeding in *Heliconius* was facilitated by

134 an enhanced, visually-orientated ‘circadian memory’ that utilizes visual landmarks  
135 (Gilbert, 1975).

136 The evolution of this behavior must involve “some elaboration of the nervous  
137 system” (Turner, 1981), suggested to lie in the mushroom bodies (Sivinski, 1989).  
138 Sivinski (1989) reported that the percentage of the brain occupied by the mushroom  
139 body in two individuals of *Heliconius charithonia* was 3–4 times larger than in six  
140 other species of butterfly, including two non-pollen feeding Heliconiini. In other  
141 insects, mushroom body expansion has been associated with increased demands for  
142 higher order information processing, either in relation to social ecology or foraging  
143 behavior, both within and between species (Dujardin, 1859; Withers et al., 1993,  
144 2008; Gronenberg et al., 1996; Ehmer and Ron, 1999; Molina and O’Donnell, 2007;  
145 Smith et al., 2010; O’Donnell et al., 2013; Gronenberg et al., 1996; Fahrbach et al.,  
146 2003; Farris and Roberts, 2005; Farris and Schulmeister, 2011).

147 Mushroom bodies have a variety of roles in olfactory associative learning,  
148 sensory integration, filtering and attention (Zars, 2000; Farris, 2005, 2013; Menzel,  
149 2014). Direct experimental evidence also links mushroom body function with spatial  
150 learning and memory in some, but not all, insects (Mizunami et al., 1998; Neuser et  
151 al., 2008; Ofstad et al., 2011). Comparisons across species suggest that extreme  
152 evolutionary expansion of the mushroom body is commonly associated with changes  
153 in foraging behavior that depend on spatial memory or the complexity of sensory  
154 information utilized by the species (Farris, 2005, 2013). For example, in  
155 Hymenoptera, mushroom body expansion coincides with the origin of parasitoidism  
156 (Farris and Schulmeister, 2011), a dietary adaptation that involves place-centered  
157 foraging and spatial memory for host location (Rosenheim, 1987; van Nouhuys and  
158 Kaartinen, 2008).

159 Patterns of ontogenetic neuropil plasticity in trap-lining insects, such as the  
160 honeybee, *Apis mellifera*, further link foraging behavior and the mushroom bodies  
161 (Withers et al., 1993; Durst et al., 1994; Capaldi et al., 1999; Farris et al., 2001).  
162 Honeybees show two forms of post-eclosion growth in mushroom body volume; age  
163 dependent growth, which occurs regardless of environmental variation, and  
164 experience dependent growth which increases with foraging or social experience  
165 (Withers et al., 1993; Durst et al., 1994; Fahrbach et al., 1998, 2003; Farris et al.,  
166 2001; Maleszka et al., 2009). A similar pattern is found in other Hymenoptera, and  
167 there is an intriguing correspondence between the rate and timing of mushroom body

168 growth and the onset of foraging behavior (Gronenberg et al., 1996; Kühn-Bühlmann  
169 and Wehner, 2006; Withers et al., 2008; Jones et al., 2013). These combined growth  
170 processes involve substantial volumetric changes, typically 20–30% increases from  
171 emergence to maturity (Gronenberg et al., 1996; Fahrbach et al., 1998; Jones et al.,  
172 2013), which most probably have strong biological and behavioral significance. Age  
173 and environmental effects on neuropil growth are also found in some other central and  
174 peripheral neuropil (Gronenberg et al., 1996; Jones et al., 2013). Whether *Heliconius*  
175 show similar ontogenetic profiles or experience dependent plasticity in mushroom  
176 body growth, as might be predicted if they are involved in spatial memory, is not  
177 known.

178 Assessing levels of environment dependent neurological plasticity may provide  
179 insights into the role behavior may have played during the *Heliconius* radiation, and  
180 during speciation in general (Pfennig et al., 2010; Snell-Rood, 2013). Speciation in  
181 *Heliconius* is thought to frequently mimetic shifts associated with habitat divergence (  
182 Mallet, 1993; Jiggins, 2008). Different mimicry rings represent alternative adaptive  
183 peaks, and may be associated with variation in predator communities, interspecific  
184 competition, habitat structure and/or light environment (Smiley, 1978; Mallet, 1993;  
185 Estrada and Jiggins, 2002; Merrill et al 2013). Consequently, shifts in mimetic  
186 resemblance may impose extensive secondary selection for behavioral adaptations.  
187 Indeed, several parapatric sister-species occur along habitat gradients (Jiggins et al.,  
188 1996; Estrada and Jiggins, 2002; Arias et al., 2008), supporting evidence that different  
189 mimicry rings are ecologically separated (Smiley, 1978; Boggs et al., 1981; Estrada  
190 and Jiggins, 2002). A potential role for neural plasticity is established in some cases  
191 of recent ecological divergence and adaptation (e.g Gonda et al., 2009a; b, 2013; Park  
192 and Bell, 2010). Whether habitat-shifts during the early stages of speciation in  
193 *Heliconius* are facilitated by behavioral and neurological plasticity is unknown but of  
194 considerable interest (Merrill et al., 2015).

195 In the current analysis we begin to investigate these topics. We first revisit  
196 *Heliconius* brain composition to confirm Sivinski's (1989) observation that they are  
197 greatly expanded. We then compare brain composition between recently emerged  
198 insectary-reared individuals, aged insectary-reared individuals, and wild-caught  
199 individuals to address several key questions: i) how big are *Heliconius* mushroom  
200 bodies? ii) how does their morphology compare with other trap-line foragers? iii) do  
201 they have post-eclosion growth patterns comparable to other trap-line foragers? and

202 iv) is such environmentally induced plasticity present in, or restricted to, the  
203 mushroom bodies? These intra-specific comparisons lay the groundwork for  
204 comparative analyses across Heliconiini examining the origin and timing of  
205 mushroom body expansion. In the meantime, we compare the relative investment in  
206 different neuropil in two species of *Heliconius*, *H. erato* and *H. hecale* to each other,  
207 and to other Lepidoptera to explore how selection has shaped overall brain  
208 composition.

209

## 210 MATERIALS & METHODS

211

### 212 Animals

213 We collected individuals of two species of *Heliconius*, *H. hecale melicerta* and *H.*  
214 *erato demophoon*. *H. hecale* is generally found in tall forest throughout central  
215 America and the Amazon basin, whilst *H. erato* is a widespread forest edge specialist  
216 (Brown, 1981). Wild individuals were collected from Gamboa (9°7.4' N, 79°42.2' W,  
217 elevation 60 m) and the nearby Soberanía National Park, República de Panamá. At  
218 this locality *H. hecale* belongs to the 'tiger pattern' mimicry ring, whilst *H. erato*  
219 belongs to the 'postman' mimicry ring which are at least partially segregated by  
220 habitat preference (Estrada and Jiggins, 2002). Five males and five females of each  
221 species were sampled from the wild. We assume all wild-caught individuals were  
222 sexually mature, and that the age range is not biased between species or sexes.

223 Wild individuals were compared with individuals from first or second-  
224 generation insectary-reared stock populations, descended from wild caught parents  
225 from the same sampling localities. Stock populations were kept in controlled  
226 conditions in cages (approximately 1 × 2 × 2 m) of mixed sex at roughly equal  
227 densities. Cages were housed at the *Heliconius* insectaries at the Smithsonian Tropical  
228 Research Institute's (STRI) facility in Gamboa (see:  
229 [www.heliconius.org/resources/research-facility](http://www.heliconius.org/resources/research-facility)). Stocks had access to their preferred  
230 host plant (*Passiflora biflora* and *P. vitifolia* respectively for *H. erato* and *H. hecale*),  
231 a pollen source (*Psychotria elata*) and feeders containing c. 20% sugar solution with  
232 an additional bee-pollen supplement to ensure there was a pollen excess. Larvae were  
233 allowed to feed naturally on the host plant.

234 After emergence from the pupae insectary-reared individuals were collected  
235 for two age groups, a recently emerged ‘young’ group (1–3 days post emergence) and  
236 an ‘old’ group (2–3 weeks post emergence). *Heliconius* are generally considered to  
237 undergo a “callow” period of general inactivity immediately after emergence that lasts  
238 about 5 days, during which flight behavior is weak and males are sexually inactive  
239 (Mallet, 1980). These age groups therefore represent behaviorally immature and  
240 mature individuals. In *Bombus impatiens*, which has a comparable lifespan to  
241 *Heliconius* (Plath, 1934; Benson, 1972; Ehrlich and Gilbert, 1973), age-related  
242 growth plateaus after c. 10 days (Jones et al., 2013). If *Heliconius* have a comparable  
243 developmental trajectory we would expect growth to have reached a plateau in the old  
244 group. For *H. hecale* 5 males and 5 females were sampled for both age groups, in *H.*  
245 *erato* 4 males and 6 females were sampled for the ‘young’ group and 5 males and 4  
246 females were sampled for the ‘old’ group. For samples where it was possible to  
247 measure the exact time of emergence, there is no significant difference between *H.*  
248 *hecale* and *H. erato* in age structure of the old (*H. erato*: mean = 22.6 days, SD = 8.6;  
249 *H. hecale*: mean = 26.4 days, SD = 5.5;  $t_{13} = -0.899$ ,  $p = 0.385$ ) or young (*H. erato*:  
250 mean = 1.7 days, SD = 0.8; *H. hecale*: mean = 1.3 days, SD = 1.1;  $t_{17} = 0.829$ ,  $p =$   
251 0.419) insectary-reared groups.

252 We took three body size measurements for each individual: body mass,  
253 weighted to 0.01 g using a OHAUS "Gold Pocket" pocket balance (model YA102);  
254 and body length and wingspan, measured using FreeLOGIX 6 inch digital calipers.  
255 Wings were kept as voucher specimens in glassine envelopes. Samples were collected  
256 and exported under permits SEX/A-3-12 and SE/A-7-13 obtained from the Autoridad  
257 Nacional del Ambiente, República de Panamá in conjunction with STRI.

258

### 259 **Antibodies and sera for neuropil staining**

260 We used indirect immunofluorescence staining against synapsin to reveal the neuropil  
261 structure of the brain under a confocal microscope (Ott, 2008). This technique  
262 exploits the abundant expression of synapsin, a vesicle-associated protein, at  
263 presynaptic sites. Monoclonal mouse anti-synapsin antibody 3C11 (anti-SYNORF1;  
264 (Klagges et al., 1996) was obtained from the Developmental Studies Hybridoma Bank  
265 (DSHB), University of Iowa, Department of Biological Sciences, Iowa City, IA  
266 52242, USA (RRID: AB\_2315424). The 3C11 antibody was raised against a  
267 bacterially expressed fusion protein generated by adding a glutathione S-transferase



268 (GST)-tag to a cDNA comprising most of the 5' open reading frame 1 of the  
269 *Drosophila melanogaster* synapsin gene (*Syn*, CG3985). The binding specificity of  
270 this antibody was characterised in *D. melanogaster* (Klagges et al., 1996). The  
271 epitope was later narrowed down to within LFGGMEVCGL in the C domain  
272 (Hofbauer et al., 2009). Bioinformatic analysis has confirmed the presence of this  
273 motif in Lepidopteran genomes, and demonstrated that it is highly conserved across  
274 Lepidoptera (Montgomery and Ott, 2015). 3C11 immunostaining has been used as an  
275 anatomical marker of synaptic neuropil in a wide range of arthropod species including  
276 several Lepidoptera: *Danaus plexippus* (Heinze and Reppert, 2012), *Godyris zavaleta*  
277 (Montgomery and Ott, 2015) *Heliothis virescens* (Kvello et al., 2009) and *Manduca*  
278 *sexta* (El Jundi et al., 2009b). The staining pattern obtained with 3C11 in the present  
279 analysis is similar to other Lepidoptera. Cy2-conjugated affinity-purified polyclonal  
280 goat anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, West  
281 Grove, PA) was obtained from Stratech Scientific Ltd., Newmarket, Suffolk, UK  
282 (Jackson ImmunoResearch Cat No. 115-225-146, RRID: AB\_2307343).

283

#### 284 **Immunocytochemistry**

285 Brains were fixed and stained following a published protocol (Ott, 2008) previously  
286 applied to a range of invertebrates including the Monarch butterfly, *D. plexippus*  
287 (Heinze and Reppert, 2012), and the Zavaleta Glasswing, *G.* (Montgomery and Ott,  
288 2015). The protocol was divided into two stages, the first of which was performed at  
289 the STRI Gamboa Field Station. Briefly, the brain was exposed under HEPES-  
290 buffered saline (HBS; 150 mM NaCl; 5 mM KCl; 5 mM CaCl<sub>2</sub>; 25 mM sucrose;  
291 10 mM HEPES; pH 7.4) and fixed in situ for 16–20 hours at room temperature (RT)  
292 in zinc-formaldehyde solution (ZnFA; 0.25% (18.4 mM) ZnCl<sub>2</sub>; 0.788% (135 mM)  
293 NaCl; 1.2% (35 mM) sucrose; 1% formaldehyde) under agitation. Fixation with ZnFA  
294 affords considerably better antibody penetration, staining intensity and preservation of  
295 morphology than conventional (para)formaldehyde fixation (Ott, 2008; Heinze and  
296 Reppert, 2012). The brain was subsequently dissected out, under HBS, by removing  
297 the eye cuticle in slices before gently plucking away the main body of the ommatidia  
298 and the basement membrane. After removing any surrounding material, the brain was  
299 lifted from the head capsule, washed 3 × in HBS and placed into 80% methanol/20%  
300 DMSO for a minimum of 2 hours under agitation. The brain was then transferred to

301 100% methanol and stored at RT. After transportation back to the UK samples were  
302 stored at -20°C.

303 In the second stage of the protocol, performed in laboratory conditions in the  
304 UK, the samples were brought to RT and rehydrated in a decreasing methanol series  
305 (90%, 70%, 50%, 30%, 0% in 0.1 M Tris buffer, pH 7.4, 10 minutes each). Normal  
306 goat serum (NGS; New England BioLabs, Hitchin, Hertfordshire, UK) and antibodies  
307 were diluted in 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 1% DMSO  
308 and 0.005% NaN<sub>3</sub> (PBSd). Non-specific antibody binding was blocked by pre-  
309 incubation in 5% NGS (PBSd-NGS) for 2 hours at RT. Antibody 3C11 was then  
310 applied at a 1:30 dilution in PBSd-NGS for 3.5 days at 4°C under agitation. The  
311 brains were rinsed in PBSd for 3 × 2 hours before applying the Cy2-conjugated anti-  
312 mouse antibody 1:100 in PBSd-NGS for 2.5 days at 4°C under agitation. This was  
313 followed by a series of increasing concentrations (1%, 2%, 4% for 2 hours each, 8%,  
314 15%, 30%, 50%, 60%, 70% and 80% for 1 hour each) of glycerol in 0.1 M Tris buffer  
315 with DMSO to 1%. The brains were then passed in a drop of 80% glycerol directly  
316 into 100% ethanol and agitated for 30 minutes; the ethanol was changed three times  
317 with 30-minute incubations. Finally, to clear the tissue, the ethanol was underlain with  
318 methyl salicylate, the brain was allowed to sink, before the methyl salicylate was  
319 refreshed twice with 30 minute incubations.

320

### 321 **Confocal imaging**

322 Samples were mounted in fresh methyl salicylate between two round cover slips  
323 separated by a thin metal washer (UK size M8 or M10). All imaging was performed  
324 on a confocal laser-scanning microscope (Leica TCS SP8, Leica Microsystem,  
325 Mannheim, Germany) at the University College London Imaging Facility, using a 10×  
326 dry objective lens with a numerical aperture of 0.4 (Leica Material No. 11506511,  
327 Leica Microsystem, Mannheim, Germany). For each individual brain we captured a  
328 series of overlapping stacks using a mechanical z-step of 2 μm with an x-y resolution  
329 of 512 × 512 pixels. Imaging the whole brain required 3×2 stacks in the x-y  
330 dimensions with an overlap of 20%. Tiled stacks were automatically merged in Leica  
331 Applications Suite Advanced Fluorescence software. Each brain was scanned from  
332 the posterior and anterior side to span the full z-dimension of the brain. These two  
333 image stacks were subsequently merged in Amira 3D analysis software 5.5 (FEI

334 Visualization Sciences Group), using a custom module ‘Advanced Merge’ which  
335 aligns images using affine registration and subsequently merges to produce the  
336 combined stacks. We manually optimized the re-sampling procedure from anterior  
337 and posterior stacks for each individual. Finally, to correct for the artifactually  
338 shortened  $z$ -dimension associated with the 10 $\times$  air objective, a correction factor of  
339 1.52 was applied to the voxel size in the  $z$ -dimension (Heinze and Reppert, 2012a;  
340 Montgomery and Ott, 2015). Images presented in the figures to illustrate key  
341 morphological details were captured separately as single confocal sections with an  $x$ - $y$   
342 resolution of 1024  $\times$  1024 pixels.

343

#### 344 **Neuropil segmentations and volumetric reconstructions**

345 Neuropils were reconstructed from the confocal image stacks in Amira 5.5. We  
346 assigned image regions to anatomical structures in the Amira *labelfield* module by  
347 defining outlines based on the brightness of the synapsin immunofluorescence. This  
348 process segments the image into regions that are assigned to each particular structure,  
349 and regions that are not. Within each stack, every forth or fifth image was manually  
350 segmented using the outline or magic-wand tool. The segmentation was then  
351 interpolated in the  $z$ -dimension across all images that contain the neuropil of interest  
352 before being fine-edited and smoothed in all three dimensions. The *measure statistics*  
353 module was used to determine volumes (in  $\mu\text{m}^3$ ) for each neuropil. 3D polygonal  
354 surface models of the neuropils were constructed from the smoothed labelfield  
355 outlines using the *SurfaceGen* module. The color code used for the neuropils in the  
356 3D models is consistent with previous neuroanatomical studies of invertebrate brains  
357 (Brandt et al., 2005; Kurylas et al., 2008; El Jundi et al., 2009a, b; Dreyer et al., 2010;  
358 Heinze and Reppert, 2012; Montgomery and Ott, 2015).

359 The whole-brain composite stacks were used to reconstruct and measure six  
360 paired neuropils in the optic lobes, and seven paired and two unpaired neuropils in the  
361 midbrain. All paired neuropils were measured on both sides of the brain in wild-  
362 caught individuals to permit tests of asymmetry, yielding two paired measurements  
363 per brain (*i.e.*,  $N = 10 \times 2$ ) for each structure. We found no evidence of volumetric  
364 asymmetry for either species ( $p > 0.05$  for each neuropil in a paired t-tests) and  
365 therefore summed the volumes of paired neuropil to calculate the total volume of that  
366 structure. In insectary-reared individuals we therefore subsequently measured the

367 volume of paired neuropil from one hemisphere, chosen at random, and multiplied the  
368 measured volume by two to obtain an estimate of total volume of that neuropil.  
369 Finally, we measured the total neuropil volume of the midbrain to permit statistical  
370 analyses that control for allometric differences. In keeping with the earlier  
371 Lepidopteran literature, we use the term ‘midbrain’ for the fused central mass that  
372 comprises of the protocerebral neuromere excluding the optic lobes, the deuto- and  
373 tritocerebral neuromeres, and the sub-esophageal neuromeres. For the following  
374 statistical analyses we analyzed the central body as a single structure, and summed the  
375 volumes of the mushroom body lobes and peduncles as the boundary between these  
376 structures was not always clear.

377

### 378 **Intraspecific statistical analyses**

379 In all statistical analyses continuous variables were  $\log_{10}$ -transformed to meet  
380 assumptions of normality. Unpaired two-tailed two-sample *t*-tests were used to test  
381 for volumetric differences between sexes or groups. We found no robust evidence of  
382 sexual dimorphism in neuropil volume of wild caught individuals that could not be  
383 explained by allometric scaling and therefore combined male and female data.  
384 However, we note that our sample size for each sex is unlikely to be sufficient to  
385 provide conclusive support either for or against sexual dimorphism.

386 Our analyses focused on two intra-specific comparisons: i) we compared  
387 ‘young’ and ‘old’ insectary-reared individuals and interpret significant differences as  
388 evidence for post-eclosion growth or delayed maturation; and ii) we compared wild-  
389 caught individuals with ‘old’ insectary-reared individuals and interpret significant  
390 differences as evidence for environmentally induced, experience dependent plasticity.  
391 These comparisons were made by estimating the allometric relationship with a  
392 measure of overall brain size for each neuropil. The standard allometric scaling  
393 relationship can be modeled as  $\log(y) = \beta[\log(x)] + \alpha$ . We used standard major axis  
394 regressions in the SMATR 3 package (Warton et al., 2012) to test for significant shifts  
395 in the allometric scaling parameter ( $\beta$ ) or the y-intercept ( $\alpha$ ). To permit comparisons  
396 between neuropil, a consistent independent variable was used throughout the analyses  
397 that accounts for allometric scaling with total brain size. This was the total volume of  
398 the midbrain minus the combined volume of all segmented neuropil in the midbrain,  
399 referred to as ‘rest of midbrain’ (rMid).

400 Significant differences in  $\beta$  suggest the proportional increase in the dependent  
401 variable with size differs between groups, i.e. the slopes are significantly different.  
402 The *slope.com* function in SMATR calculates a likelihood ratio statistic for the  
403 absence of a common slope,  $\chi^2$  distributed with one degree of freedom. Where we  
404 identified no heterogeneity in the allometric scaling parameter ( $\beta$ ) we performed two  
405 further tests. First, we tested for significant differences in  $\alpha$  that suggest discrete  
406 ‘grade-shifts’ in the relationship between two variables. The *elev.com* function in  
407 SMATR calculates a Wald statistic for the absence of shifts along the y-axis,  $\chi^2$   
408 distributed with one degree of freedom. Second, we tested for major axis-shifts along  
409 a common slope. This is indicative of co-ordinated changes in the size of the  
410 dependent and independent variable between groups. The *shift.com* function in  
411 SMATR calculates a Wald statistic for the absence of a shift along a common axis,  $\chi^2$   
412 distributed with one degree of freedom. For this test, when significant, we also report  
413 the fitted axis (FA) mean that describes the mean position of the group on the  
414 common fitted axis. For all statistically significant tests we also present the effect  
415 size, measured by the correlation coefficient ( $r$ ). Effect sizes of  $0.1 < r < 0.3$  are  
416 interpreted as ‘small’ effects,  $0.3 < r < 0.5$  ‘medium’ effects, and  $r < 0.5$  ‘large’ effects  
417 (Cohen, 1988).

418 Patterns of brain:body allometry were explored in a similar manner, using  
419 total neuropil volume as the dependent variable (summed volumes of all neuropil in  
420 the optic lobes plus the total midbrain volume), and comparing the results obtained  
421 using alternative body size measurements as the independent variable. Body mass  
422 varies with body condition and reproductive state, we therefore anticipated that body  
423 length may be a more reliable way of assessing the allometric relationship between  
424 brain and body size in wild caught individuals.

425 We complemented these analyses with a multivariate analysis of all  
426 segmented neuropil volumes and the unsegmented midbrain volume using Discrimant  
427 Function Analysis (DFA) to test how reliably individuals can be assigned to their  
428 respective groups on the basis of their volumetric differences in neuropil. In this  
429 analysis, as well as providing a percentage of correctly assigned individuals, Wilks’  
430 lambda provides a measure of the proportion of total variance not explained by group  
431 differences, and the  $\chi^2$  statistic provides a test for significant group differences. All  
432 statistical analyses were performed in R (R Development Core Team, 2008), using the

433 standard *stats* and *smatr* package, except for the DFA, which was performed in SPSS  
434 v. 22 for OS X (SPSS Inc., Chicago, IL)

435

### 436 **Interspecific statistical analyses**

437 An examination of allometric scaling was also applied to interspecific analyses  
438 between *H. hecale* and *H. erato* to test for species differences in brain composition.  
439 We compared both wild-caught and ‘old’ insectary-reared individuals. Where  
440 significant species differences are found in wild caught individuals but not ‘old’  
441 insectary-reared individuals we interpret this as evidence of species-dependent  
442 environmental effects, perhaps associated with differences in habitat or light-  
443 environment preference. Where significant species differences are found for both  
444 comparisons we interpret this as evidence of heritable differences in brain  
445 development.

446 We collected published data for neuropil volumes of four other Lepidoptera;  
447 the Monarch butterfly (*D. plexippus*; Heinze and Reppert, 2012), the Zavaleta Glass  
448 wing (*Godyris zavaleta*; Montgomery and Ott, 2015), the Giant Sphinx moth  
449 (*Manduca sexta*; El Jundi et al., 2009b) and the Tobacco Budworm moth (*Heliothis*  
450 *virescens*; Kvello et al., 2009). Data were available for eight neuropils across all four  
451 species. We calculated the relative investment in each neuropil by comparing its  
452 volume to the total neuropil volume of either the whole brain (excluding the lamina,  
453 which was not measure in *Heliothis virescens*), or of only the midbrain. Relative size  
454 was measured by calculating the residuals from a phylogenetically-corrected least  
455 squares (PGLS) linear regression between each structure and the rest of the brain  
456 performed in BayesContinuous in BayesTraits (freely available from  
457 [www.evolution.rdg.ac.uk](http://www.evolution.rdg.ac.uk); Pagel, 1999). For this analysis, a phylogeny of the six  
458 species was created using data on two loci, *COI* and *EF1a* (GenBank Accession IDs,  
459 *COI*: EU069042.1, GU365908.1, JQ569251.1, JN798958.1, JQ539220.1,  
460 HM416492.1; *EF1a*: EU069147.1, DQ157894.1, U20135.1, KC893204.1,  
461 AY748017.1, AY748000.1). The data were aligned and concatenated using MUSCLE  
462 (Edgar, 2004), before constructing a maximum likelihood tree in MEGA v.5 (Tamura  
463 et al., 2011). Differences in brain architecture across species were visualised by  
464 multivariate Principal Component Analysis of these data, and visualized as biplots  
465 (Greenacre, 2010) in R package *ggbiplot* (V.Q. Vu, <https://github.com/vqv/ggbiplot>).

466 Finally, we extended our phylogenetic analysis across insects using a similar  
467 approach. We restricted this analysis to volumetric data collected with similar  
468 methodology (Rein et al., 2002; Brandt et al., 2005; Kurylas et al., 2008; Dreyer et al.,  
469 2010; Ott and Rogers, 2010; Wei et al., 2010) as it is not known how comparable data  
470 collected with alternative fixing, staining and imaging methods are. The phylogenetic  
471 relationship of these insects was taken from Trautwein et al. (2012).

472

## 473 RESULTS

474

### 475 **General layout of the *Heliconius* brain**

476 In general, the overall layout and morphology of the *Heliconius* brain (Fig. 1) is  
477 similar to that of other Lepidoptera (El Jundi et al., 2009; Kvello et al., 2009; Heinze  
478 and Reppert, 2012a; Montgomery and Ott, 2015). The midbrain forms a single medial  
479 mass, containing the supra-esophageal ganglion to which the sub-esophageal ganglion  
480 is fused. Synapsin immunostaining effectively labeled regions of synaptic neuropil,  
481 with minimal fluorescence in fiber tracts and cell bodies, permitting segmentation of  
482 six paired neuropils in the optic lobes, and eight paired and two unpaired neuropils in  
483 the midbrain. Together with the rest of the midbrain (rMid), which lacks distinct  
484 internal boundaries, we measured the volumes of these neuropils in 59 individuals  
485 across both species (Table 1). In the following we briefly describe the main  
486 anatomical features of the sensory neuropils, the central complex and the mushroom  
487 bodies in wild individuals. We then present the results of intra-specific comparisons  
488 between age groups, between wild and insectary-reared individuals, before moving to  
489 an analysis of inter-specific comparisons between *H. hecale* and *H. erato*, and across  
490 a wider taxonomic scale.

491

### 492 **Sensory neuropil**

493 As expected for a strongly visual, diurnal butterfly, the optic lobes (OL; Fig. 2A–H)  
494 account for a large proportion of total brain volume (approximately 64%). As is the  
495 case in both *D. plexippus* and *G. zavaleta* the lamina (La), two-layered medulla (Me)  
496 (Fig. 2E), accessory medulla (aMe), lobula (Lob) and lobula plate (Lop) are well  
497 defined and positioned in the OL as nested structures, running lateral to medial  
498 (Fig. 2A). The La has a distinct, brightly stained inner rim (iRim; Fig. 2E), a feature

499 common to all diurnal butterflies analyzed thus far (Heinze and Reppert, 2012;  
500 Montgomery and Ott, 2015). In common with *D. plexippus* we identify a thin strip of  
501 irregularly shaped neuropil running ventrally from the aME to the Me (Fig. 2G–H).

502 We also identify a sixth neuropil in the OL that we believe to be homologous  
503 to the optic glomerulus (OG; Fig. 2B,F) identified in *D. plexippus* (Heinze and  
504 Reppert, 2012), which is absent in other Lepidopteran brains described to date and  
505 was postulated to be Monarch-specific. As in *D. plexippus* this neuropil is a multi-  
506 lobed, irregularly shaped structure positioned to the medial margin of the Lob with  
507 which it appears to be connected. In *Heliconius* the OG is not as extended in the  
508 anterior margin as *D. plexippus* and is subsequently confined to the OL, without  
509 protrusion into the optic stalk or midbrain (Fig. 2A,B,F). The position of the OG in  
510 *Heliconius* is also similar to that of a much smaller neuropil observed in *G. zavaleta*  
511 (Montgomery and Ott, 2015). We suggest these structures may be homologous but  
512 differentially expanded.

513 The midbrain contains two further neuropils with primary functions in  
514 processing sensory information; the anterior optic tubercle (AOTu), a visual center,  
515 and the antennal lobe (AL), the primary olfactory neuropil. We can identify all four  
516 components of the AOTu previously described in *D. plexippus* and *G. zavaleta*  
517 butterflies (Heinze and Reppert, 2012; Montgomery and Ott, 2015); the small, closely  
518 clustered nodular unit (NU), strap (SP) and lower unit (LU), and the much larger  
519 upper unit (UU) (Fig. 2C). As in other butterflies, the UU is expanded compared with  
520 nocturnal moths (El Jundi et al., 2009; Kvello et al., 2009). The proportion of total  
521 neuropil comprised of the AOTu is, however, larger in *D. plexippus* (0.736%) than  
522 *Heliconius* (0.400% in *H. hecale* and 0.368% in *H. erato*).

523 The AL are innervated by the antennal nerves and comprise the central fibrous  
524 neuropil (CFN) around which small, round glomeruli are arranged (Figure 3A,B). In  
525 *Heliconius* the AL comprises 2% of the total brain neuropil volume, and contains  
526 approximately 68 glomeruli (estimated in one individual of each sex: *H. erato* ♂ = 69,  
527 ♀ = 68; *H. hecale* ♂ = 68, ♀ = 67). This is similar to estimates for other Lepidoptera,  
528 which generally range between 60 and 70 (Boeckh and Boeckh, 1979; Rospars, 1983;  
529 Berg et al., 2002; Huetteroth and Schachtner, 2005; Masante-Roca et al., 2005; Skiri  
530 et al., 2005; Kazawa et al., 2009; Heinze and Reppert, 2012; Carlsson et al., 2013;  
531 Montgomery and Ott, 2015), and the number of olfactory receptor genes (70)  
532 identified in the *H. melpomene* genome (Dasmahapatra et al., 2012). We observe no



533 expanded macro-glomeruli or obvious candidates for sexual dimorphic glomeruli  
534 suggesting an absence of any MGC-like structure in *Heliconius*. This is in keeping  
535 with all diurnal butterflies described to date (Rosparis, 1983; Heinze and Reppert,  
536 2012; Carlsson et al., 2013), with the exception of the more olfactorily orientated *G.*  
537 *zavaleta* in which the AL is 5% of total neuropil volume and a sexually dimorphic  
538 MGC is observed (Montgomery and Ott, 2015).

539 We took advantage of comparable datasets for *H. erato*, *H. hecale* and *G.*  
540 *zavaleta* to further investigate the significance of changes in AL size by testing  
541 whether changes in relative AL volume are due to an increased volume of glomeruli  
542 or CFN. The former would reflect heightened olfactory sensitivity, as larger glomeruli  
543 receive more projections from sensory neurons, while the latter may indicate changes  
544 in the number or complexity of local interneurons. We find evidence for a grade-shift  
545 in the allometric relationship between total glomerular volume and midbrain volume  
546 when *G. zavaleta* is compared to either *H. erato* (Wald  $\chi^2 = 10.709$ ,  $p = 0.001$ ) or *H.*  
547 *hecale* (Wald  $\chi^2 = 9.139$ ,  $p = 0.003$ ) (Fig. 3C, circles). We also identify a grade-shift  
548 between CFN volume and midbrain volume in *G. zavaleta* over *H. erato* (Wald  $\chi^2 =$   
549  $30.282$ ,  $p < 0.001$ ) and *H. hecale* (Wald  $\chi^2 = 26.638$ ,  $p < 0.001$ ) (Fig. 3C, triangles).  
550 Hence, AL expansion in *G. zavaleta* is due to changes in both CFN and glomerular  
551 volume. However, when comparing CFN and glomerular volume directly against one  
552 another we again find a significant grade-shift in *G. zavaleta* over *H. erato* (Wald  $\chi^2 =$   
553  $19.680$ ,  $p < 0.001$ ) and over *H. hecale* (Wald  $\chi^2 = 31.663$ ,  $p < 0.001$ ) demonstrating  
554 greater CFN volume, relative to glomerular volume, in *G. zavaleta* (Fig. 3D). This  
555 suggests variation in Lepidopteran AL size may be largely explained by changes in  
556 the complexity of olfactory processing, which may in turn explain the consistency in  
557 glomerular number across species with contrasting diel patterns (see above).

558

### 559 **Central complex**

560 The central complex is a multimodal integration center linked to a range of functions  
561 from locomotor control to memory (Pfeiffer and Homberg, 2014). Within the  
562 limitations of the current analysis, the anatomy of the *Heliconius* central complex  
563 shows strong conservation with *D. plexippus* and *G. zavaleta* (Heinze and Reppert,  
564 2012; Montgomery and Ott, 2015). The central body (CB) is positioned along the  
565 midline of the central brain and is formed of two neuropils, the upper (CBU) and  
566 lower (CBL) divisions, which are associated with small paired neuropils, the noduli

567 (No), located ventrally to the CB (Fig. 4A–D,G). Two further paired neuropils, the  
568 protocerebral bridge (PB; Fig. 4A,E) and posterior optic tubercles (POTu; Fig. 4A,F),  
569 are positioned towards the posterior margin of the brain.

570

### 571 **Mushroom bodies**

572 The most striking aspect of *Heliconius* brain morphology are the hugely expanded  
573 mushroom bodies, previously noted by Sivinski (1989). These neuropils span the  
574 depth of the brain along the anterior-posterior axis (Fig. 5A–K; Fig. 6A,A'). On the  
575 anterior side, the mushroom body lobes (MB-lo) lie above the AL. As in *D. plexippus*  
576 (Heinze and Reppert, 2012a) the distinct lobe structure observed in moths (El Jundi et  
577 al., 2009; Kvello et al., 2009) is lost, possibly due to extensive expansion. The only  
578 identifiable feature is a lobe curving round the medial margin, likely to be part of the  
579 vertical lobe (Fig. 5D,F). The MB-lo merges with a long cylindrical neuropil, the  
580 pedunculus (MB-pe) that extends to the posterior midbrain and is comprised of  
581 several twisted layers of neuropil. The boundary between the MB-lo and MB-pe is not  
582 distinct. The combined volume of the MB-lo+pe accounts for 12.2% of total midbrain  
583 volume in *H. hecale* and 14.6% of total midbrain volume in *H. erato*, at least twice  
584 that reported for other Lepidoptera (Sjöholm et al., 2005; El Jundi et al., 2009; Kvello  
585 et al., 2009; Heinze and Reppert, 2012a; Montgomery and Ott, 2015). At the posterior  
586 end, the MB-pe splits into two roots that are encircled by the mushroom body calyx  
587 (MB-ca; Fig. 5A,H,K). A Y-tract runs parallel to the MB-pe from the posterior  
588 boundary of the MB-lo to the junction between the MB-pe and MB-ca. The Y-tract  
589 ventral loblets seen in other Lepidoptera (El Jundi et al., 2009; Kvello et al., 2009) are  
590 not distinct, presumably having merged with the MB-lo (Fig. 5A,J,N).

591 The MB-ca is a deeply cupped, un-fused, double-lobe structure (Fig. 5A,C).  
592 Two concentric zones can be identified (Fig. 5E), though the boundary is not distinct  
593 throughout the depth of the neuropil. The MB-ca comprises 20.7% and 23.9% of total  
594 midbrain volume in *H. hecale* and *H. erato* respectively, at least three times greater  
595 than reported in other Lepidoptera (Sjöholm et al., 2005; El Jundi et al., 2009; Kvello  
596 et al., 2009; Heinze and Reppert, 2012a; Montgomery and Ott, 2015). In some  
597 individuals the MB-ca is so large that it protrudes into the OL resulting in a distortion  
598 of shape caused by constriction around the optic stalk (Fig. 5H). We also observe  
599 some degree of pitting in the posterior surface of the MB-ca (Fig. 5I). This pitting is

600 related to radially arranged columnar domains that are apparent within the calycal  
601 neuropil (Fig. 5J,K,M)

602 Below the junction between MB-pe and MB-ca is a brightly stained globular  
603 neuropil with a poorly defined anterior margin (Fig. 5M). It is possible this is an  
604 accessory calyx, which has a sporadic phylogenetic distribution across Lepidoptera  
605 and other insects (Farris, 2005). However, accessory calyces are generally positioned  
606 closely to the MB-pe/MB-ca, as is observed in *D. plexippus* (Heinze and Reppert,  
607 2012). This neuropil also lacks the ‘spotty’ appearance of the accessory calyx in *D.*  
608 *plexippus* (Heinze and Reppert, 2012). We therefore do not believe this is an  
609 accessory calyx. Similar ‘satellite’ neuropils that are near, but not directly linked to  
610 the mushroom bodies are observed in other insects, for example *Neuroptera* (Farris,  
611 2005). One potentially interesting observation is that this neuropil lies in a position  
612 roughly equivalent to the anterior end of the OG in *D. plexippus* (Heinze and Reppert,  
613 2012). Although generally unclear, in some individuals it is possible to follow a  
614 narrow, weakly stained fiber tract from the medial margin of the Lob/OG to this  
615 position, via an area of relatively intense staining in the optic stalk (Fig. 5L). It is  
616 possible this neuropil is functionally connected to the OG, and that OG expansion  
617 along the anterior margin in *D. plexippus* occurred along the path of this pre-existing  
618 connection, resulting in a single, elongated neuropil. In other insects the MB-pe also  
619 receives afferent innervation from regions of the protocerebrum other than the MB-ca  
620 (Schürmann, 1970; Li and Strausfeld, 1997, 1999). A pronounced fiber tract  
621 emanating from the AOTu UU clearly runs against the dorsal boundary of the MB-pe  
622 (Figure 5O), but whether or not there is a functional connection, that might suggest  
623 the integration of processed visual information with the MB-pe/lo, is unclear.

624

### 625 **Brain:body allometry**

626 The relationship between total neuropil and body mass is not significant at  $p > 0.05$   
627 for either wild *H. hecale* (log-log SMA regression,  $p = 0.055$ ) or wild *H. erato* ( $p =$   
628  $0.863$ ). However, as expected body mass is more variable than either body length or  
629 wingspan (*H. hecale*, relative SD of body mass = 15.32%, body length = 10.43%,  
630 wingspan = 9.08%; *H. erato*, relative SD of body mass = 19.00%, body length =  
631 5.98%, wingspan = 4.59%), most likely due to differences in feeding and reproductive  
632 state. When either body length or wingspan is used as the measure of body size a  
633 significant association is recovered both in *H. hecale* (body length  $p = 0.020$ ,

634 wingspan  $p = 0.019$ ) and in *H. erato* (body length  $p = 0.011$ , wingspan  $p = 0.010$ ). We  
635 subsequently used body length to compare the relationships between total neuropil  
636 volume and body size between groups.

637 We identified a significant grade-shift, between the young and old groups of  
638 both *H. erato* and *H. hecale*, in the scaling relationship between total neuropil and  
639 body length (*H. hecale*: Wald  $\chi^2 = 5.780$ ,  $p = 0.016$ ; *H. erato*: Wald  $\chi^2 = 10.124$ ,  $p =$   
640  $0.001$ ). However, there is no significant difference in body length for either species  
641 between old and young insectary-reared individuals (*H. hecale*  $t_{18} = -0.918$ ,  $p = 0.371$ ;  
642 *H. erato*  $t_{17} = 0.581$ ,  $p = 0.568$ ) suggesting the effect is primarily driven by an  
643 increase in neuropil volume. Indeed, there is a significant difference in total neuropil  
644 volume between the young and old insectary-reared age groups in *H. erato* ( $t_{17} =$   
645  $5.153$ ,  $p < 0.001$ ,  $r = 0.708$ ; Fig. 7A). This difference is observed for both total  
646 midbrain volume ( $t_{17} = 4.192$ ,  $p = 0.001$ ,  $r = 0.713$ ) and total OL volume ( $t_{17} = 5.076$ ,  
647  $p < 0.001$ ,  $r = 0.776$ ; Fig. 7A). In *H. hecale* a significant difference between young  
648 and old individuals is only observed for midbrain volume ( $t_{18} = 3.054$ ,  $p = 0.007$ ,  $r =$   
649  $0.595$ ), but not OL volume ( $t_{18} = 0.280$ ,  $p = 0.783$ ) or total neuropil volume ( $t_{18} =$   
650  $1.082$ ,  $p = 0.293$ ; Fig. 7D).

651 In *H. hecale*, the total neuropil is also 40% larger in wild caught individuals  
652 than in old insectary-reared individuals ( $t_{17} = 2.553$ ,  $p = 0.020$ ,  $r = 0.526$ ) driven by a  
653 significant difference in midbrain volume ( $t_{17} = 3.658$ ,  $p = 0.002$ ,  $r = 0.664$ ), but not  
654 OL volume ( $t_{18} = 1.728$ ,  $p = 0.101$ ; Fig. 7D). We also do not observe a similar  
655 difference in body length between wild and old insectary-reared individuals (*H.*  
656 *hecale*  $t_{18} = 0.983$ ,  $p = 0.436$ ). Although this does not result in a grade-shift between  
657 wild and old insectary-reared individuals for body length and total neuropil volume  
658 (Wald  $\chi^2 = 2.058$ ,  $p = 0.151$ ), we do observe a grade-shift when midbrain is analysed  
659 separately (Wald  $\chi^2 = 4.725$ ,  $p = 0.030$ ) No significant volume or size differences are  
660 found between wild and old insectary-reared individuals in *H. erato* (total neuropil:  $t_{17}$   
661  $= -0.432$ ,  $p = 0.671$ ; midbrain:  $t_{17} = -0.732$ ,  $p = 0.474$ ; OL:  $t_{17} = -0.123$ ,  $p = 0.904$ ;  
662 body length:  $t_{17} = 1.009$ ,  $p = 0.327$ ; Fig. 7A).

663

664

### 665 **Post-eclosion growth in the volume of individual neuropil regions**

666 Evidence of significant age-effects on volume was uncovered for many neuropil  
667 regions in both species, indicating an important role for post-eclosion brain

668 maturation. In *H. erato* volumetric differences between the age groups are  
669 widespread, with only the OG failing to show a significant expansion in old  
670 individuals (Table 2A). There is some evidence for age-related differences in the  
671 allometric scaling coefficients for aMe and PB, and for grade-shifts in OG and POTu,  
672 but these are weak relative to the strong major axis shifts observed for all segmented  
673 neuropils (Table 2A). The largest shifts are observed for the POTu (difference in  
674 fitted-axis (FA) mean = 0.604), aME (difference in FA mean 0.536), MB-ca  
675 (difference in FA mean = 0.496) and MB-lo+ped (difference in FA mean = 0.393;  
676 Fig. 6A-C).

677 In *H. hecale* old insectary-reared individuals have significantly larger absolute  
678 midbrain volumes ( $t_{18} = 3.054$ ,  $p = 0.007$ ,  $r = 0.584$ ) but not OL volumes ( $t_{18} = 1.728$ ,  
679  $p = 0.101$ ). However, not all segmented midbrain neuropil show the same expansion;  
680 the rMid, components of the mushroom body complex, central complex and AL are  
681 all significantly larger in old individuals, but the AOTu, POTu and all optic lobe  
682 neuropil are not (Table 2B). Neuropil expansion appears to occur in a co-ordinated  
683 manner, maintaining the allometric relationship between the segmented neuropil and  
684 rMid (Table 2B). The only exceptions are the La, Me and OG, which show significant  
685 grade-shifts resulting in a reduced volume of these neuropil relative to rMid volume  
686 in old individuals. All other segmented neuropil show major-axis shifts along a  
687 common slope towards higher values in old individuals (Table 2B). The largest shifts  
688 are observed for the MB-ca (difference in fitted-axis mean = 0.279) and MB-lo+ped  
689 (difference in fixed axis mean = 0.250; Fig. 6A'-C').

690

### 691 **Experience-dependent plasticity in neuropil volume**

692 The strong contribution of post-emergence growth to brain maturation in *Heliconius*  
693 provides a potential window during which the environment could influence the size of  
694 different neuropil. We compared wild and old insectary-reared individuals to test for  
695 the presence of such environment-dependent neural plasticity. A clear signature of  
696 environmentally induced volumetric differences is found in both species, but the  
697 pattern differs between them.

698 In *H. erato* wild individuals do not have significantly larger absolute neuropil  
699 volumes for any measured trait (Table 3A). However, several neuropils show  
700 evidence of differences in allometric scaling or grade-shifts between wild and old  
701 insectary-reared individuals. The neuropil affected by altered scaling coefficients ( $\beta$ )

702 include the MB-ca, the Lop, and the PB, all of which result shallower scaling  
703 relationships with rMid volume in wild caught individuals (Table 3A; Figure 7B,C).  
704 The MB-lo+ped is the only neuropil to show an unambiguous grade-shift whilst  
705 maintaining a common slope, and also shows a major axis shift (difference in FA  
706 mean = 0.250; Fig. 6B).

707 In *H. hecale* wild individuals have significantly larger total midbrains ( $t_{18} =$   
708 3.658,  $p = 0.002$ ). The only segmented neuropil to reflect this difference are the MB-  
709 ca and MB-lo+ped (Table 3B; Fig. 6A'-C'), while the rMid is also larger in wild  
710 individuals ( $t_{18} = 3.417$ ,  $p = 0.003$ ). For example, the average MB-ca volume of old  
711 insectary-reared individuals is only 68.3% of the average wild MB-ca volume, for the  
712 young insectary-reared individuals it is 49.3% (Figure 6A',C'). For MB-lo+pe these  
713 figures are 76.9% and 58.7% respectively (Figure 6A',B'). For comparison, in *H.*  
714 *erato* the average MB-ca volume of old insectary-reared individuals is 96.2% of the  
715 average wild MB-ca volume, for the young insectary-reared individuals it is 59.7%  
716 (Fig. 6A-C). For MB-lo+pe these figures are 96.9% and 63.9% respectively  
717 (Fig. 6A-C).

718 The only neuropil in the optic lobes to differ significantly volume in *H. hecale*  
719 is the Me. The allometric relationship between neuropil volumes and rMid differs for  
720 all neuropil either in the allometric scaling coefficient or the intercept, except for the  
721 mushroom body components and aMe.(Table 3A; Figure 7E,F). However, for aME  
722 this pattern is caused by a lack of allometric scaling in insectary-reared individuals  
723 (SMA  $p = 0.552$ ). The mushroom bodies show evidence of a major axis shift along a  
724 common slope (difference in FA mean MB-ca = 0.355, MB-lo+ped = 0.299). Given  
725 all grade-shifts result in smaller neuropil volumes relative to rMid (Fig. 7E,F) volume  
726 we interpret this as indicating the rMid and mushroom bodies show coordinated  
727 environment-dependent increases in volume whilst other neuropil volumes remain  
728 largely constant, but with subsequently altered allometric relationships with rMid.

729 These results highlight potential differences in the maturation and plasticity of  
730 brain size and structure between *H. hecale* and *H. erato*. Most notably the rMid  
731 appears to have greater amounts of environment-dependent growth in *H. hecale*  
732 which results in altered scaling relationships with other neuropil. In *H. hecale* this is  
733 accompanied by coordinated expansion of the mushroom body components, which, in  
734 volumetric terms is less pronounced in *H. erato*. Instead, the mushroom bodies, along

735 with PB and LoP, are relatively larger in wild *H. erato* due to a grade-shift in the  
736 scaling relationship with rMid.

737

### 738 **Divergence in brain composition between *H. hecale* and *H. erato***

739 *H. hecale* is significantly larger than *H. erato* for all body size measurements (wild  
740 caught: body mass  $t_{17} = 7.262$ ,  $p < 0.001$ ; body length  $t_{17} = 5.442$ ,  $p < 0.001$ ; wingspan  
741  $t_{17} = 6.071$ ,  $p < 0.001$ ). *H. hecale* also have larger midbrain ( $t_{17} = 2.713$ ,  $p = 0.014$ ,  $r =$   
742  $0.539$ ) and OL volumes ( $t_{17} = 2.866$ ,  $p = 0.010$ ,  $d = 1.351$ ). This difference does not,  
743 however, match the body size difference, resulting in a grade-shift in the allometric  
744 relationship between body length and total neuropil volume between the species  
745 (Wald  $\chi^2 = 5.695$ ,  $p = 0.017$ ; Fig. 7A). This is predominantly driven by a grade shift  
746 in OL:body allometry (Wald  $\chi^2 = 8.257$ ,  $p = 0.004$ ; Fig. 8B) rather than in  
747 midbrain:body allometry (Wald  $\chi^2 = 3.805$ ,  $p = 0.051$ ; Fig. 8C). Hence, *H. erato* have  
748 larger OL volumes relative to body size than *H. hecale*. The same pattern is observed  
749 in old insectary-reared individuals suggesting this is not an environmentally induced  
750 difference (midbrain: Wald  $\chi^2 = 1.721$ ,  $p = 0.189$ ; OL Wald  $\chi^2 = 11.131$ ,  $p < 0.001$ ).

751 Among segmented neuropils only the La and Me show significant absolute  
752 volumetric differences between wild-caught individuals of the two species, both being  
753 larger in *H. hecale* (Table 4A). Analyses of allometric relationships imply species  
754 differences in neuropil investment (Table 4A). La and Me show strong major-axis  
755 shifts and we identify evidence for grade-shifts between rMid and aMe, Lob, LoP and  
756 OG, as well as all segmented neuropil in the midbrain except the PB and POTu (Table  
757 4A). In all of these cases the shift is towards larger relative volumes in *H. erato*, and  
758 may be partially driven by the differences in rMid volume which is significantly  
759 larger in *H. hecale* ( $t_{17} = 3.582$ ,  $p = 0.002$ ,  $r = 0.656$ ). Only one neuropil, the AL,  
760 shows evidence for a difference in the allometric coefficient  $\beta$  between species. The  
761 AL scales with a significantly higher coefficient in *H. erato* ( $\beta = 1.197$ , 95% C.I. =  
762  $0.699$ – $2.05$ ) than *H. hecale* ( $\beta = 0.470$ , 95% C.I. =  $0.313$ – $0.705$ ), indicating the ALs  
763 of *H. erato* show a greater proportional increase in size as rMid increases than in *H.*  
764 *hecale*.

765 Species differences between old insectary-reared individuals are notably less  
766 widespread (Table 4B). The La is the only neuropil with a significant absolute  
767 volumetric difference between species, and notably rMid volume is also not

768 significantly different ( $t_{17} = -1.391$ ,  $p = 0.181$ ). There is no evidence for species  
769 differences in scaling coefficients for AL, and grade-shifts are restricted to Lam, Me,  
770 and OG. However, the direction of the grade-shifts indicates *H. hecale* have relatively  
771 larger component volumes. The difference in results between wild and old insectary-  
772 reared individuals is most probably explained by species differences in environment-  
773 dependent changes in rMid volume. The expanded rMid volume in wild individuals  
774 masks the grade-shifts observed in La and Me in old insectary-reared individuals and,  
775 because other segmented neuropil do not show a similar expansion, lead to the wide-  
776 spread grade-shifts observed between wild caught *H. hecale* and *H. erato*. The effect  
777 is even sufficient to reverse the direction of grade-shift for CB and LoP. These results  
778 imply role for environmentally induced plasticity in contributing to species  
779 differences, but also indicate environment-independent differences exist.

780 Discrimant Function Analysis (DFA) of component volumes is able to  
781 distinguish wild individuals of the two species along one major axis, correctly  
782 assigning 100% of samples to the correct species (Fig. 8D). Wilks Lambda suggests  
783 group differences explain 90% of total variance between samples, although the formal  
784 statistical test for a group difference does not reach significance ( $\chi^2 = 20.76$ , 14  
785 degrees of freedom,  $p = 0.108$ ). To test whether phenotypic differentiation between  
786 species is caused by both environmentally independent and dependent variation we  
787 performed an additional DFA using four data-groups: i) wild *H. erato*, ii) old  
788 insectary-reared *H. erato*, iii) wild *H. hecale*, iv) old insectary-reared *H. hecale*. This  
789 analysis reduced the variation along three axes (Table 5A,B; Fig. 8E,F) which assign  
790 87.2% of individuals to the correct data-group. No individuals were assigned to the  
791 wrong species, incorrect assignment always occurred between wild and old groups of  
792 the same species.

793 We subsequently performed a MANOVA to test for whether DF1, DF2 and  
794 DF3 are associated with species and/or group (wild vs. old), and to test for significant  
795 species-group interactions (Table 6C). DF1 showed a significant association with both  
796 species and group, DF2 and DF3 were specifically associated with group. Only DF2  
797 showed a significant species by group interaction. Visual inspection of Discrimant  
798 Function plots (Fig. 8E,F) suggests DF2 separates wild and old insectary-reared  
799 individuals more effectively in *H. hecale*.

800

801 **Divergence in brain composition across Lepidoptera and other insects**



802 Beyond *Heliconius* there is a clear signature of adaptive divergence in the  
803 composition of Lepidoptera brains. We combined the data collected for the present  
804 analysis with comparable volumetric data for eight neuropils from four other species  
805 (as collated in Montgomery and Ott, 2015, Table 3; Fig. 9A). After correcting for  
806 allometric scaling, using phylogenetically-corrected regressions against total neuropil  
807 volume, the six species can be separated along two principal components that together  
808 explain 90.7% of variance. PC1 is heavily loaded by sensory neuropil in the negative  
809 direction, and the MB-ca and MB-lo+ped in the positive direction (Table 6). PC2 is  
810 heavily loaded by the Me in the positive direction and the AL and CB in the negative  
811 direction. This separates the six species into three pairs, representing (i) *H. hecale* and  
812 *H. erato*; (ii) the other diurnal butterflies, *D. plexippus* and *G. zavaleta*; and (iii) the  
813 night-flying moths, *H. virescens* and *M. sexta* (Fig. 9B). When midbrain neuropil are  
814 analyzed separately, PC1, which explains 68.7% of variance, marks an axis  
815 dominated by the AL, CB and MB components. PC2, which explains an additional  
816 23.3% variance, is strongly loaded by the AOTu (Fig. 9C). This leads to two clusters  
817 grouping (i) *H. hecale* and *H. erato*, which invest heavily in mushroom body neuropil,  
818 and (ii) the night-flying moths and *G. zavaleta*, which invest heavily in olfactory  
819 neuropil; leaving *D. plexippus* isolated by its large AOTu volume. Although this  
820 could be partially explained by the AOTu appearing small in *Heliconius* due to the  
821 expanded mushroom bodies, when the MB volume is removed from total midbrain,  
822 *D. plexippus* still has the largest residual AOTu size, suggesting this is a genuine  
823 signal of divergence. Whether the larger AOTu volume in *D. plexippus* is derived, or  
824 more typical for diurnal butterflies, is not known.

825 Finally, we consider the most notable adaptation in *Heliconius* brains, the  
826 increase in mushroom body volume, in a wider phylogenetic context. The combined  
827 volume of the calyx, pedunculus and lobes accounts for 13.7% of total brain neuropil  
828 volume in *H. erato*, and 11.9% in *H. hecale*. This is much larger than reported for any  
829 other Lepidoptera measured with similar methods (range 2.3-5.1%). Expressed as a  
830 percentage of the midbrain, to remove the effects of variation in the large OL, *H.*  
831 *erato* (38.5%) and *H. hecale* (32.9%) again exceed other Lepidoptera (4.8-13.5%) by  
832 3–7 fold. These figures are also much larger than reported for *H. charithonia* (4.2% of  
833 total brain size) by Sivinski (1989), whose figures for other Lepidoptera are also  
834 much lower suggesting the difference is probably explained by variation in  
835 methodology. However, Sivinski reported the mushroom bodies of *H. charithonia* to

836 be approximately 4-times larger than those of the other butterflies measured, which is  
837 similar to the fold differences we observe here.

838 Comparisons beyond Lepidoptera are complicated by differences in the  
839 neuropil measured. For example, the total neuropil volume is not reported for the  
840 *Tribolium* (Dreyer et al., 2010), *Leucophaea* (Wei et al., 2010) or *Drosophila* (Rein et  
841 al., 2002) brains, which instead report individual neuropil volumes as a percentage of  
842 the sum of all segmented components. The most well matched comparisons are to  
843 *Apis mellifera* (Brandt et al., 2005) and *Schistocerca gregaria* (Kurylas et al., 2008)  
844 for which mushroom body volume and midbrain volume are reported (Fig. 9D). Even  
845 here, however, the data is not fully comparable, as the SOG is not fused with the  
846 midbrain in *S. gregaria*. In *A. mellifera* the mushroom bodies comprise 65.4% of the  
847 midbrain, (40.6% MB-ca, 24.8% MB-lo+ped) (Brandt et al., 2005), in gregarious-  
848 phase *S. gregaria* they comprise 15.1% (8.2% MB-ca including the accessory calyx,  
849 6.3% MB-lo+ped) (Kurylas et al., 2008). Ott and Rogers (2010) report proportional  
850 calyx volumes in *S. gregaria* of 7.6% and 8.6% in solitary-phase and gregarious-  
851 phase locusts, respectively (both figures include the accessory calyx), but did not  
852 measure the peduncle. In terms of raw volume our estimates (Table 1) *Heliconius*  
853 mushroom bodies are roughly equal in size to *A. mellifera*.

854 More general comparisons can be made expressing mushroom body size as a  
855 percentage of segmented neuropil (Me+Lobula system, CB, MB and AL) that were  
856 labeled across a wider range species (Dreyer et al., 2010). In this analyses *A.*  
857 *mellifera*, *T. castaneum* and *L. maderana* all devote a larger proportion of total neuropil  
858 to the mushroom bodies than *Heliconius*. However, they also have substantially  
859 smaller optic lobes. If one instead examines the ratio of percentage mushroom body  
860 volume to the percentage of the two other midbrain neuropil (AL and CB), *Heliconius*  
861 have the largest ratios (*H. erato*: 6.4; *H. hecale*: 6.7) by some way, exceeding even *A.*  
862 *mellifera* (3.8). Similarly, the residuals from the highly significant ( $p < 0.001$ ) PGLS  
863 regression (Fig. 9E) between percentage OL and percentage MB for *Heliconius* (*H.*  
864 *erato* +8.2; *H. hecale* +7.5) are only exceeded by *A. mellifera* (+11.9), which both far  
865 exceed the forth-largest residual (*S. gregaria* +2.2). A similar result is found  
866 comparing percentage MB to percentage AL+CB. This fairly crude analysis at least  
867 demonstrates that *Heliconius* rank highly across insects in terms of investment in  
868 mushroom body neuropil.

869

## 870 DISCUSION

871

872 We have described the layout and volume of the major neuropils in the brain of two  
873 species of *Heliconius* butterflies. We have further demonstrated patterns of variation  
874 across age groups of insectary-reared and wild individuals that are consistent with  
875 significant post-eclosion growth and experience-dependent plasticity in neuropil  
876 volume. Our analyses confirm previous reports of a substantial expansion in  
877 mushroom body size during the evolution of *Heliconius* butterflies (Sivinski, 1989).  
878 Indeed, our data suggest this previous work underestimated the proportional size of  
879 the *Heliconius* mushroom bodies. We further demonstrate levels of plasticity in  
880 mushroom body volume comparable to those found in foraging insects known for  
881 their capacity to learn spatial information (e.g. Withers et al., 1993, 2008; Gronenberg  
882 et al., 1996; Fahrbach et al., 1998, 2003; Maleszka et al., 2009; Jones et al., 2013).  
883 However, plasticity is not limited to the mushroom bodies, nor is the signal of  
884 adaptive divergence in neuropil structure. In the following, we discuss how and why  
885 *Heliconius* may have evolved such large mushroom bodies, what the convergent  
886 expansion of the mushroom body in independent insect lineages can tell us about their  
887 function and significance, and finally widen our scope to consider how the brains of  
888 different Lepidoptera are adapted to their different ecological niches.

889

### 890 *Mushroom body expansion in Heliconius*

891 As a percentage of total brain volume, or indeed as a raw volume, *Heliconius* have the  
892 largest mushroom body so far reported in Lepidoptera (Sivinski, 1989; Sjöholm et al.,  
893 2005; Rø et al., 2007; Kvello et al., 2009; Snell-Rood et al., 2009; Dreyer et al., 2010;  
894 Heinze and Reppert, 2012b; Montgomery and Ott, 2015). The expansion affects the  
895 calyx, pedunculus and lobes. The calyx has an expanded double-lobe, deeply cupped  
896 structure, superficially reminiscent of the mushroom body calyx of *A. mellifera*  
897 (Brandt et al., 2005), but structurally quite different from the calyx of *Danaus*  
898 *plexippus* which is composed of a series of concentric sub-structures that are not  
899 deeply cupped. As in *D. plexippus* (Heinze and Reppert, 2012) and to a lesser extent  
900 *Godyris zavaleta* (Montgomery and Ott, 2015), the lobe system is sufficiently  
901 expanded to merge individual lobes that are distinct in *Manduca sexta* and *Heliothis*  
902 *virescens* (Sjöholm et al., 2005; Rø et al., 2007; El Jundi et al., 2009; Kvello et al.,

903 2009). This precludes an accurate assessment of whether all lobes are equally  
904 expanded with the current data, which would be of interest given their relationship to  
905 different aspects of memory (Krashes et al., 2007; Guven-ozkan and Davis, 2014;  
906 Stopfer, 2014). It appears likely however that both the  $\alpha'/\beta'$  and  $\alpha/\beta$  lobes, which are  
907 responsible for consolidating long-term memory, and for the retrieval and expression  
908 of these memories (Guyen-ozkan and Davis, 2014), contribute to the expansion.

909 Mushroom body expansion suggests the presence of selection for greater  
910 memory capacity during *Heliconius* evolution. The unique pollen-feeding behavior of  
911 adult *Heliconius*, and associated demands of foraging for spatially distributed  
912 resources, provides the most likely source of this selection pressure (Gilbert, 1975;  
913 Sivinski, 1989). Several studies have reported evidence of spatially and temporally  
914 faithful foraging patterns (Ehrlich and Gilbert, 1973; Gilbert, 1975, 1993; Mallet,  
915 1986) comparable with the well described trap-lining behavior of foraging bees  
916 (Janzen, 1971; Heinrich, 1979). In Hymenoptera, this behavior involves landmark  
917 based spatial memory (Cartwright and Collett, 1983; Dyer, 1991; Menzel et al.,  
918 2005). Mushroom bodies are implicated in spatial memory both through experimental  
919 manipulation (Mizunami et al., 1998) and comparative neuro-ecological studies  
920 (Farris and Schulmeister, 2011), although the role selection for spatial memory played  
921 in mushroom body expansion in Hymenoptera remains unproven for some authors  
922 (Menzel, 2014).

923 Comparisons across *Heliconius* and non-pollen feeding genera in the  
924 Heliconiini may provide a test of this hypothesis. Sivinski (1989) reported that two  
925 individuals of *Dione juno* and *Dryas iulia*, both non-pollen feeding allies to  
926 *Heliconius*, have mushroom bodies within the range of other Lepidoptera. This  
927 provides preliminary support that mushroom body expansion may have occurred co-  
928 incidentally with a single origin of pollen feeding at the base of *Heliconius*. However,  
929 several genera were not sampled, including the specious genus *Euides* which is most  
930 closely related to *Heliconius* (Beltrán et al., 2007; Kozak et al., 2015). As such,  
931 further sampling is required to confirm this conclusion. This work is underway. It is  
932 also conceivable alternative selection pressures may play a role, such as the degree of  
933 host-plant specialization (Brown, 1981) or the evolution of social roosting (Benson,  
934 1972; Mallet, 1986). It is possible these factors are inter-related, as *Passiflora* may be  
935 incorporated into trap-lines between pollen plants (Gilbert, 1975, 1993), and the  
936 sedentary home-range behavior required for trap-lining may predispose *Heliconius* to

937 sociality (Mallet, 1986). Indeed, Farris and Schulmeister (2010) suggest sociality in  
938 hymenoptera is an exaptation, dependent on the co-option of a pre-existing  
939 elaboration of the mushroom bodies in response to the demands for spatial foraging in  
940 parasitic Euhymenopteran. Similar hypotheses have been proposed in vertebrates, for  
941 example, the initial expansion of the primate brain may have been driven by visual  
942 specialization (Barton, 1998). Social intelligence may therefore be viewed as an  
943 exaptation of this initial expansion that facilitated processing of a greater range and  
944 complexity of information (Barton and Dunbar, 1997).

945 It is certainly likely that pollen feeding at least plays a role in meeting the  
946 energetic costs of increased neural investment, even if it does not explain its origin.  
947 Increased investment in both the neural tissue required for learning-based foraging  
948 (Aiello et al., 1995; Laughlin et al., 1998; Snell-Rood et al., 2009) and the act of  
949 learning itself (Mery and Kawecki, 2004; Burger et al., 2008; Snell-Rood et al., 2009,  
950 2011) will impose significant energetic costs, potentially resulting in trade-offs  
951 against other traits (Dukas, 1999; Burns et al., 2011; Snell-Rood et al., 2011; Snell-  
952 Rood, 2013). The fitness benefits of pollen-feeding lie in increased longevity without  
953 reproductive senescence (Dunlap-Pianka et al., 1977), facilitating a shift in the  
954 energetic costs of chemical defense to larvae as adults are no longer dependent on  
955 larval fat body stores (Cardoso and Gilbert, 2013), and amino acid transfer to eggs  
956 (O'Brien et al., 2003). These combined effects must outweigh the energetic costs  
957 incurred by greater neuropil investment.

958

### 959 ***Post-eclosion growth in mushroom body volume***

960 *Heliconius* are not as robust to environmental perturbation, or as amenable as  
961 experimental subjects, as honeybees and ants. As a result our conditions are perhaps  
962 less controlled than comparable studies in these insects. Nevertheless, there is a clear  
963 effect of age on mushroom body size (Fig. 6). In both species, the mushroom bodies  
964 are significantly larger in aged individuals. These volume differences of 38.0% for the  
965 calyx and 34.0% for the lobe system in *H. erato*, and 27.9% for the calyx and 23.7%  
966 for the lobe system in *H. hecale* are comparable, if not greater than, the effects seen in  
967 Hymenoptera (e.g. c. 30% in *Camponotus floridanus* (Gronenberg et al., 1996); c.  
968 20% in *Bombus impatiens* (Jones et al., 2013)).

969 Our comparisons between aged insectary-reared individuals and wild caught  
970 individuals also identify experience-dependent plasticity. This 'experience' in the

971 wild likely includes a greater range of movement as well as a greater complexity of  
972 foraging, and more variable environmental conditions and social interactions. In both  
973 species we see evidence for mushroom body plasticity, however it is notable that the  
974 pattern differs between species. In *H. hecale* a strong volumetric difference is found  
975 between old insectary-reared and wild caught individuals for both the calyx (32%  
976 difference) and lobes (24% difference). Again, this is a result of a major-axis shift in  
977 the unsegmented midbrain. This is not just an effect of increases in total brain size, as  
978 no other neuropil shows a similar increase resulting in widespread grade-shifts  
979 towards smaller relative size in wild caught individuals. This may suggest a  
980 coordinated pattern of growth between the mushroom bodies and unsegmented areas  
981 of the midbrain or alternatively independent plasticity in the two structures. In *H.*  
982 *erato* the effect of environmental plasticity is somewhat different. Although we find  
983 similar volumes in old insectary-reared individuals and wild caught individuals, both  
984 the mushroom body calyx and lobe system show allometric grade-shifts resulting in  
985 greater volumes relative to the unsegmented midbrain in wild individuals compared to  
986 insectary-reared individuals. It is also notable that this grade-shift is apparent because,  
987 in contrast to *H. hecale*, *H. erato* does not show a concomitant increase in midbrain  
988 volume in wild individuals.

989 It is currently unclear what causes this species difference. The difference may  
990 reflect changes in connectivity between the mushroom bodies and neuropil housed in  
991 the unsegmented midbrain, or perhaps experience-dependent plasticity in unrelated  
992 midbrain structures. Alternatively, the difference may be a sampling artifact.  
993 Although the ages of the insectary-reared individuals are not significantly different,  
994 we cannot rule out that the age structure of wild-caught individuals is biased. A  
995 further possibility is that *H. erato* and *H. hecale* respond differently to conditions in  
996 captivity, perhaps due to contrasting natural behavior in the wild. Nevertheless, our  
997 observation that *Heliconius* mushroom bodies show similar levels of post-eclosion  
998 growth to Hymenoptera, and a similar two-phase pattern of environmentally  
999 independent and dependent growth, provides further evidence of evolutionary  
1000 convergence with *Heliconius*.

1001 These large, volumetric changes in mushroom body size presumably have  
1002 some significance on how the mushroom bodies are functioning. Some insight into  
1003 the functional significance may be gained from investigating what is causing the  
1004 increase in volume. Ongoing proliferation of Kenyon cells seems unlikely, if

1005 neurogenesis is restricted to the larval and pupal stages, as it is in Hymenoptera  
1006 (Masson, 1970; Fahrbach et al., 1995). Instead the increased volume may indicate  
1007 changes in dendritic growth, as is suggested for mushroom body plasticity in  
1008 Hymenoptera (Gronenberg et al., 1996; Farris et al., 2001). Farris et al. (2001)  
1009 demonstrated age and experience dependent plasticity in dendrite branching. The  
1010 former reflecting developmentally programmed growth of extrinsic neuron processes  
1011 into the mushroom bodies, and the latter reflecting an expansion in the complexity of  
1012 Kenyon cell processes. The resulting changes in dendritic fields may indicate altered  
1013 neural connectivity.

1014 Finally, although the mushroom bodies show the strongest and most consistent  
1015 effects, it is also striking that plasticity, and in particularly age related growth, is not  
1016 restricted to the mushroom bodies. This has been observed in other insects (Kühn-  
1017 Bühlmann and Wehner, 2006; Snell-Rood et al., 2009; Ott and Rogers, 2010; Smith et  
1018 al., 2010; Heinze and Florman, 2013; Jones et al., 2013). As in previous examples  
1019 significant age-dependent effects in *Heliconius* appear to play a role in the  
1020 development of sensory neuropil involved in the processing of both visual and  
1021 olfactory information. This may not be unexpected as sensory neuropil do not merely  
1022 relay information on to the central brain, but also process information *in situ*  
1023 (Muscedere et al., 2014). Both the medulla and lobula system are implicated in  
1024 processing different types of visual information (Paulk et al., 2009a; b). Similarly,  
1025 odor learning may be due to plasticity in the antennal lobes themselves rather than, or  
1026 as well as, higher order structures (Hammer and Menzel, 1998; Rath et al., 2011). We  
1027 also find evidence of plasticity in the central complex. In Lepidoptera, the plasticity in  
1028 central body has been demonstrated in response to experience with novel host plants  
1029 (Snell-Rood et al., 2009), whilst the protocerebral bridge increases in volume  
1030 following migratory experience in *D. plexippus* (Heinze et al., 2013). Our results  
1031 suggest *Heliconius* may provide a useful system within which to explore the  
1032 behavioral relevance of plasticity in the mushroom bodies, and other structures.

1033

#### 1034 ***Convergent expansion of mushroom bodies: similarities and differences***

1035 Extensive mushroom body expansion is now reported in lineages of four insect  
1036 orders; Dictyoptera, herbivorous Scarabaeidae, Hymenoptera, and *Heliconius* (Farris,  
1037 2013). We see three ecological traits that are shared by at least two of these lineages:  
1038 dietary adaptations, central place foraging, and sociality. In scarab beetles mushroom

1039 body expansion is associated with a shift from coprophagy to a generalist  
1040 phytophagous diet, possibly in response to increased behavioral flexibility in the  
1041 context of foraging (Farris and Roberts, 2005). It is conceivable a similar selection  
1042 pressure may apply to cockroaches. In contrast, the initial increase in mushroom body  
1043 size coincides with the origin of a specialist foraging behavior in Hymenoptera,  
1044 parasitoidism (Farris and Schulmeister, 2011). As discussed above, the expansion of  
1045 the *Heliconius* mushroom bodies may also be related to a specialist foraging behavior.  
1046 In these cases, central place foraging may have facilitated the origin of more social  
1047 behavior (Mallet, 1986; Farris and Schulmeister, 2011), which may have secondarily  
1048 led to further increases in mushroom body volume (Dujardin, 1859; Withers et al.,  
1049 1993; Gronenberg et al., 1996; Ehmer and Ron, 1999). The common theme of these  
1050 hypotheses is that mushroom body expansion evolves in response to the need for  
1051 parsing a greater complexity of environmental information, facilitating the emergence  
1052 of new behaviors (Chittka and Niven, 2009).

1053 Two anatomical traits associated with mushroom body expansion provide  
1054 support for this hypothesis: increased complexity of sensory input into the mushroom  
1055 bodies, and subdivision of the mushroom body calyx. In addition to olfactory inputs,  
1056 the mushroom body calyx receives direct input from the optic lobes in Hymenoptera  
1057 and phytophagous Scarab beetles (Gronenberg, 2001; Farris and Roberts, 2005; Farris  
1058 and Schulmeister, 2011). This increase in functional inputs is reflected in the  
1059 subdivision of the calyx into the lip, which processes olfactory information, and the  
1060 collar and basal ring, which process visual information (Gronenberg and Hölldobler,  
1061 1999). This suggests mushroom body expansion may be partly caused by the  
1062 acquisition of new functions (Farris, 2013).

1063 It is not known whether *Heliconius* receive visual input into the mushroom  
1064 bodies. However, this has been demonstrated in species of both butterflies (Snell-  
1065 Rood et al., 2009) and moths (Sjöholm et al., 2005), and some anatomical features of  
1066 the *Heliconius* brain suggest the possibility that this does occur. It is notable,  
1067 however, that *Heliconius* lack clear subdivision of the calyx as seen in Hymenoptera.  
1068 In contrast, Heinze and Reppert (2012) described clear structural subdivision, or  
1069 zonation, that they postulated may be analogous to the *A. mellifera* lip, collar and  
1070 basal ring. We do not interpret the lack of clear zonation in *Heliconius* as evidence  
1071 that there is no functional sub-division, as *Spodoptera littoralis* displays localization of  
1072 visual processing in the mushroom body calyx that is not apparent without labeling



1073 individual neurons. Given the implied role for visual landmark learning in *Heliconius*  
1074 foraging behavior (Jones, 1930; Gilbert, 1972, 1975; Mallet, 1986), it seems probable  
1075 that there has been some integration of visual information processing with the  
1076 mushroom bodies.

1077 In other species the mushroom body also receives input relaying gustatory and  
1078 mechanosensory information (Schildberger, 1983; Homberg, 1984; Li and Strausfeld,  
1079 1999; Farris, 2008). These may also be of relevance in *Heliconius* given the  
1080 importance of gustatory and mechanosensory reception in host-plant identification  
1081 (Schoonhoven, 1968; Renwick and Chew, 1994; Briscoe et al., 2013) and pollen  
1082 loading (Krenn and Penz, 1998; Penz and Krenn, 2000), although it should be noted  
1083 that there is currently no evidence these behaviors are learnt (Kerpel and Moreira,  
1084 2005; Salcedo, 2011; Silva et al., 2014).

1085

#### 1086 ***Ecological adaptations in Lepidopteran brain composition***

1087 The mushroom body is not the only neuropil to display an adaptive signature of  
1088 divergence. Our interspecific analysis across Lepidoptera strongly suggest a mosaic  
1089 pattern of brain evolution (Barton and Harvey, 2000) in response to a species' specific  
1090 ecological needs (Barton et al., 1995; Huber et al., 1997; Gronenberg and Hölldobler,  
1091 1999b; Montgomery and Ott, 2015). This pattern is particularly noticeable in the  
1092 sensory neuropil (Fig. 9B). The relative volume of the visual neuropil closely reflects  
1093 diel activity patterns, whilst the size of the antennal lobe also appears to be strongly  
1094 associated with activity pattern or a low-light diurnal niche. This is illustrated in a  
1095 PCA of midbrain neuropil (Fig. 9C) which clusters the olfactorily driven butterfly *G.*  
1096 *zavaletta* with night-flying moths (Montgomery and Ott, 2015). As expected,  
1097 *Heliconius* are distinguished by their enlarged mushroom bodies, whilst *D. plexippus*  
1098 is isolated along an axis of variation heavily loaded by the AOTu. This may reflect  
1099 dependence on visual information typical of diurnal butterflies; however, removal of  
1100 the mushroom bodies from the analysis does not explain this difference. It is possible  
1101 therefore that AOTu expansion is particularly prominent in *D. plexippus* as suggested  
1102 by Heinze and Reppert (2012). The AOTu plays a key role in relaying segregated  
1103 visual pathways (Pfeiffer et al., 2005; Heinze and Reppert, 2011; Mota et al., 2011;  
1104 Pfeiffer and Kinoshita, 2012), suggesting a plausible link between AOTu expansion  
1105 and migratory behavior (Heinze and Reppert, 2012a; Heinze et al., 2013).

1106           These macroevolutionary patterns of divergence between genera are mirrored  
1107 to some extent in comparisons between *H. erato* and *H. hecale*. In Panama, *H. hecale*  
1108 and *H. erato* differ in host-plant use (Merrill et al., 2013), and *H. hecale* are biased  
1109 towards collecting large grained pollen whereas *H. erato* collect pollen from plant  
1110 species that produce smaller grains (Estrada and Jiggins, 2002). Although this may  
1111 reflect foraging differences, it is likely that the difference is largely driven by  
1112 competitive exclusion of *H. erato* from a generally preferred pollen source (Estrada  
1113 and Jiggins, 2002) as there is no difference in the efficiency of handling different  
1114 pollen sizes in captivity (Penz and Krenn, 2000), or evidence that *H. erato*  
1115 preferentially collect small pollen grains in other wild populations (Boggs et al., 1981;  
1116 Cardoso, 2001). These data suggest *H. erato* and *H. hecale* may be using their  
1117 environment differently, and therefore be exposed to contrasting conditions and  
1118 selection pressures.

1119           Focusing on wild caught individuals, where variation in neuropil volume may  
1120 be most ecologically relevant, *H. hecale* have a significantly larger total brain size.  
1121 This is largely due to the optic lobes, and undefined regions of the midbrain. At the  
1122 level of specific neuropil only the lamina and medulla differ significantly between  
1123 species. However, interpreting allometric grade-shifts between species is complicated  
1124 by significant differences in unsegmented midbrain volume, which is used as the  
1125 independent variable throughout. In old insectary-reared individuals the significant  
1126 difference in lamina volume persists, suggesting this may be a non-plastic species  
1127 difference, whilst the effects on midbrain volume may be environment dependent.  
1128 This supports recent data showing divergence in corneal facet number and diameter  
1129 which suggests visual adaptations in different micro-habitats may play a role in  
1130 *Heliconius* diversification (Seymoure et al., in review).

1131           The behavioral relevance of divergence and plasticity in unsegmented areas of  
1132 the midbrain, which lack clear boundaries, is less obvious. Given its role in  
1133 controlling the mandibles, proboscis and mouth parts (Rehder, 1989; Bowdan and  
1134 Wyse, 2000), all of which are involved in pollen loading (Krenn and Penz, 1998; Penz  
1135 and Krenn, 2000), it is possible the effect is largely driven by variation in the sub-  
1136 esophageal ganglion caused by species differences in pollen handling in the wild. The  
1137 development of phenotypic markers that permit consistent segmentation of different  
1138 areas of the midbrain across species (e.g. Heinze and Reppert, 2012) in future studies  
1139 will permit a test of this hypothesis.

1140 Interspecific differences between *H. erato* and *H. hecale* in brain component  
1141 volumes are sufficient to correctly group individuals by species in a discriminant  
1142 function analysis. Further analysis of species and group (wild vs. old insectary-reared)  
1143 suggest both environment independent and dependent variation contributes to these  
1144 species differences. The potential role of phenotypic plasticity in facilitating  
1145 ecological diversification by bridging fitness valleys is attracting renewed and  
1146 growing interest (Pfennig et al., 2010; Snell-Rood, 2013). The numerous closely  
1147 related sister-species of *Heliconius* that exist along environmental gradients (Jiggins  
1148 et al., 1996; Jiggins and Mallet, 2000; Estrada and Jiggins, 2002; Arias et al., 2008)  
1149 may provide an opportunity to test the importance of neural phenotypic plasticity in  
1150 facilitating ecological shifts during speciation.

1151

### 1152 ***Conclusions and future prospects***

1153 We have described the layout and size of the major neuropil in two species of  
1154 *Heliconius* butterflies. This has confirmed a previous report that this genus has  
1155 dramatically expanded mushroom bodies (Sivinski, 1989). Moreover our estimates of  
1156 the percentage of the brain occupied by the mushroom bodies suggest the initial  
1157 values underestimated the size of the mushroom body. Indeed, in some comparisons,  
1158 *Heliconius* rank among the top insect species in terms of investment in mushroom  
1159 body neuropil. Through comparisons across different age groups, and between wild  
1160 and insectary-reared individuals, we further demonstrate levels of plasticity  
1161 comparable to hymenoptera, extensively studied models of mushroom body  
1162 expansion and plasticity. Our interspecific analysis reveals patterns of divergence in  
1163 brain composition between genera, and within *Heliconius*, that suggest a close  
1164 correspondence to ecological variables. This analysis lays the groundwork for  
1165 comparative and experimental analyses that will seek to dissect the costs, benefits,  
1166 behavioral relevance and proximate basis of differential expansion in key neuropil.

1167

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1175

### 1176 **Conflict of interest statement**

1177 The authors declare no conflict of interest.

1178

### 1179 **Role of authors**

1180 All authors read and approved the final manuscript. Study conception: SHM. Study  
1181 design and preliminary experiments: SHM, RMM, SRO. Fieldwork and insectary  
1182 rearing: SHM, RMM. Acquisition of data, analysis, interpretation and initial  
1183 manuscript draft: SHM. Final interpretation and drafting: SHM, RMM, SRO.

1184

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## 1602 **Abbreviations**

AL	antennal lobe
aMe	accessory medulla
AN	antennal nerve
AOTu	anterior optic tubercle
CB	central body
CBL	lower central body
CBU	upper central body
CFN	central fibrous neuropil of <i>AL</i>
DMSO	dimethyl sulphoxide
Glom	glomeruli
HBS	HEPES-buffered saline
iMe	inner medulla
iRim	inner rim of the lamina
La	lamina
LAL	lateral accessory lobes
Lo	lobula
LoP	lobula plate
LU	lower unit of <i>AOTu</i>
MB	mushroom body
MB-ca	mushroom body calyx
MB-lo	mushroom body lobes
MB-pe	mushroom body peduncle
MB-lo+pe	mushroom body lobes and peduncle combined
MBr	midbrain
Me	medulla
MGC	macro-glomeruli complex
NGS	normal goat serum
no	noduli
NU	nodule unit of <i>AOTu</i>
oMe	outer medulla
OR	olfactory receptor
OGC	optic glomerular complex
PA	pyrrolizidine alkaloids
PB	protocerebral bridge
PC	principal component
POTu	posterior optic tubercle
rMid	rest of midbrain
SP	strap of <i>AOTu</i>
UU	upper unit of <i>AOTu</i>
ZnFA	Zinc-Formaldehyde solution

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## 1605 **Figure Legends**

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### 1607 **Figure 1: Overview of the anatomy of the *Heliconius* brain.**

1608 3D models of *H. erato* (A–G) and *H. hecale* (A'–G'). **B–D** and **B'–D'**: Volume  
1609 rendering of synapsin immunofluorescence showing the surface morphology of the  
1610 brain neuropil from the anterior (A/A'), posterior (B/B'), and dorsal (C/C') view. **E–G**  
1611 and **E'–G'**: Surface reconstructions of the major neuropil compartments from the  
1612 anterior (D), posterior (E), and dorsal (F) view. Neuropil in yellow-orange: visual  
1613 neuropil, green: central complex, blue: antennal lobes, red: mushroom bodies. See  
1614 Figures 2–4 for further anatomical detail. The individuals displayed are male. Images  
1615 in A/A' are from Warren et al. (2013). Scale bars = 25 mm in A/A'; 500  $\mu$ m in B–  
1616 D/B'–D'.

1617

### 1618 **Figure 2: Anatomy of the sensory neuropils.**

1619 Images A–H are from male *H. hecale*. **A**: Surface reconstructions of the optic lobe  
1620 neuropils viewed from anterior (left image ) and posterior (right image). They  
1621 comprise the lamina (La), the medulla (Me), and accessory medulla (aMe), the lobula  
1622 (Lo), the lobula plate (LoP) and the optic glomerulus (OG). **B**: Surface reconstruction  
1623 of the optic glomerulus (OG) viewed along the anterior-posterior axis (top) and an  
1624 anterior view (bottom). **C**: Surface reconstruction of the anterior optic tubercle  
1625 (AOTu). **D–J**: Synapsin immunofluorescence in single confocal sections of the optic  
1626 lobe of *H. hecale*. **D**: Horizontal section showing all four major optic lobe neuropils  
1627 (La, Me, Lo, LoP). **E**: Frontal section showing the inner rim (iRim) of the lamina, a  
1628 thin layer on its inner surface that is defined by intense synapsin immunofluorescence.  
1629 Synapsin immunostaining also reveals the laminated structure of the medulla with two  
1630 main subdivisions, the outer and inner medulla (oMe, iMe). **F**: The OG is located  
1631 medially to the Lo; frontal section, the midbrain (MBr) occupies the left half of the  
1632 frame. **G,H**: Frontal sections showing a small, irregular neuropil (ir) observed  
1633 running from the anterior-ventral boundary of the aME as in *D. plexippus* (Heinze and  
1634 Reppert, 2012).

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1638 **Figure 3: Anatomy of the antennal lobe**

1639 **A:** 3D reconstruction of individual antennal lobe (AL) glomeruli superimposed on a  
1640 volume rendering of the anterior surface of the midbrain. **B:** Synapsin  
1641 immunofluorescence in a single frontal confocal section showing the glomeruli  
1642 (Glom) surrounding the central fibrous neuropil (CFN). Images A–B are from male  
1643 *H. hecale*. **C,D:** Allometric grade-shifts between Glom (circles) or CFN (triangles)  
1644 volume and unsegmented midbrain volume (C), and between Glom and CFN volume  
1645 (D) in *G. zavaleta* (solid blue), *H. erato* (black filled with red) and *H. hecale* (orange  
1646 filled with yellow). Scale bars = 500  $\mu\text{m}$  in A; 50  $\mu\text{m}$  in B,C,G,H; 100  $\mu\text{m}$  in B–F, J;  
1647 200  $\mu\text{m}$  in I.

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1649 **Figure 4: Anatomy of the central complex**

1650 **A/A':** Surface reconstruction of the central complex from an anterolateral (A) and  
1651 oblique posteroventral (A') view, showing the upper and lower subunit of the central  
1652 body (CBU, CBL), the noduli (No), the protocerebral bridge (PB) and posterior optic  
1653 tubercles (POTu). **B–G:** Synapsin immunofluorescence in single confocal sections. **B:**  
1654 Horizontal section showing the upper and lower subunit of the CB in relation to the  
1655 antennal lobes (AL) and the calyx (MB-ca) and pedunculus (MB-pe) of the  
1656 mushroom body. **C,D:** Frontal confocal sections at the level of the CBL (C) and CBU  
1657 (D); the CB subunits are flanked by the profiles of the vertically running MB-pe on  
1658 either side. **E:** Frontal section showing the location of the PB ventrally to the MB-ca.  
1659 **F:** POTu positioned ventrally to the MB-ca in a frontal section. **G:** Frontal section  
1660 showing position of the paired No ventrally to CBL and CBU. All images are from a  
1661 male *H. hecale*. Scale bars = 100  $\mu\text{m}$  in B–D, G; 50  $\mu\text{m}$  in E,F.

1662

1663 **Figure 5: Anatomy of the mushroom body**

1664 **A–C:** Surface reconstruction of the mushroom body viewed (A), orthogonal to the  
1665 anterior-posterior axis from a medial vantage point level with the peduncle; (B), from  
1666 anterior; and (C), from posterior. The main components are the calyx (MB-ca) shown  
1667 in dark red, and the peduncle (MB-pe) and lobes (MB-lo) shown in bright red. A Y-  
1668 tract, shown in magenta, runs parallel and slightly medial to the MB-pe. **D–K:**  
1669 Synapsin immunofluorescence in individual confocal sections. **D:** anterior view of the  
1670 midbrain showing the MB-lo, an asterik indicates the probably ventral lobe, otherwise  
1671 the individual lobes and loblets of the MB-lo are fused. **E:** Frontal section at a

1672 posterior level near the end of the MB-pe, showing the profiles of the MB-ca with its  
1673 zonation into an outer and a medial ring. **F,G** and **J,K**: Horizontal confocal sections  
1674 through the midbrain at increasing depths from dorsal towards ventral, showing MB  
1675 structure in relation to neighboring neuropil: the anterior optic tubercle (AOTu in  
1676 F,G); the antennal lobe (AL in G,J); and the central body upper division (CBU in K).  
1677 **H**: An example of a female *H. erato* where the MB-ca is deformed due expansion into  
1678 the optic lobe and constriction (C) at the optic stalk by the neural sheath surrounding  
1679 the brain. **I**: Pitted surface of the MB-ca in a very posterior tangential horizontal  
1680 section. The pitting is related to what appear to be columnar domains within the calyx  
1681 neuropil (*cf.* MB-ca in J,K,M). **L**: Areas of intense synapsin staining in the optic stalk  
1682 (OS\*); Lo, lobula; OG, optic glomerulus. **M**: Frontal section near the base of the  
1683 calyx (MB-ca) showing a satellite neuropil (sat.) located near to the MB-pe. **N**: A Y-  
1684 tract runs parallel with, and dorsally and slightly medially to the MB-pe; both are seen  
1685 in profile in this frontal section. **O**: A fiber bundle (fb) connected to the AOTu  
1686 running near the junction between the MB-pe and MB-lo. With the exception of I, all  
1687 images are from a male *H. hecale*. Scale bars A-G, J-K = 200  $\mu\text{m}$ , H-I, L-O = 100  $\mu\text{m}$ .  
1688

### 1689 **Figure 6: Age and environment dependent growth of the mushroom bodies**

1690 Surface reconstruction of the mushroom body viewed along the anterior-posterior axis  
1691 for wild-caught, old and young insectary-reared individuals of *H. erato* (**A**) and *H.*  
1692 *hecale* (**A'**). Representative individuals were chosen as those closest to the group  
1693 mean volume. Scale bar = 200  $\mu\text{m}$ . **B-C/B'-C'**: allometric relationships between MB-  
1694 lo+pe (B/B'), or MB-ca (C/C'), and the volume of the unsegmented midbrain (rMid)  
1695 for *H. erato* (B/C) and *H. hecale* (B'/C'). Data for wild caught individuals are in  
1696 green, data for old insectary-reared individuals in dark blue, and data for young  
1697 insectary-reared individuals are in light blue. Allometric slopes for each group are  
1698 shown, the slope, intercepts and major-axis means are compared in Table 2, 3.

1699

### 1700 **Figure 7: Age and environment dependent growth of brain components**

1701 **A,D**: Comparisons of raw volumes of total neuropil, total OL neuropil, total midbrain  
1702 neuropil between wild-caught, old and young insectary-reared individuals of *H. erato*  
1703 (**A**) and *H. hecale* (**D**). Significance of pair-wise comparisons are shown along the x-  
1704 axis (young-old = orange; old-wild = dark red; n.s. =  $p > 0.05$ , \* =  $p < 0.05$ , \*\* =  $p$   
1705  $< 0.01$ , \*\*\* =  $p < 0.001$ ). **B**: Allometric scaling of LoP in *H. erato*. **C**: Allometric

1706 scaling of PB in *H. erato*. **E:** Allometric scaling of OG in *H. hecale*. **F:** Allometric  
1707 scaling of CB in *H. hecale*. Note in E and F the shifts in allometry occur along the x-  
1708 axis, this is explained by the large difference in unsegmented midbrain volume  
1709 observed between wild-caught and old insectary-reared individuals in *H. hecale* as  
1710 displayed in D.

1711

1712 **Figure 8: Divergence in brain structure between *H. erato* and *H. hecale*.**

1713 **A–C:** Species differences in brain-body scaling between *H. erato* (red) and *H. hecale*  
1714 (orange). Grade-shifts towards larger neuropil volume relative to body length are seen  
1715 for total neuropil (A) but are mainly driven by OL neuropil (B) rather than midbrain  
1716 neuropil (C). **D:** Discriminant Function analysis of segmented neuropil volumes and  
1717 the unsegmented midbrain volume separates wild individuals of the two species along  
1718 a single axis (yellow, orange ring: wild *H. hecale*; red, black ring: wild *H. erato*). **E,**  
1719 **F:** DFA including wild and old insectary-reared individuals of both species (solid  
1720 orange = old insectary-reared *H. hecale*; solid black: old insectary-reared *H. erato*). E  
1721 displays DF1 vs. DF2 to illustrate that DF1 accounts for the most variation between  
1722 species, F displays DF2 vs. DF3 to illustrate that these axes separate wild and old  
1723 insectary-reared individuals of the same species.

1724

1725 **Figure 9: Divergence in brain structure across Lepidoptera, and in mushroom**  
1726 **body size across insects.**

1727 **A:** Phylogenetic relationships of Lepidoptera (red branches) and other insects (grey  
1728 branches) for which comparable data are available. Branches are not drawn  
1729 proportional to divergence dates, numbers refer to labels in panel E. **B,C:** Principal  
1730 Component analysis of segmented neuropil volumes, corrected for allometric scaling  
1731 with the unsegmented midbrain and phylogeny. **B:** Analysis using all neuropil. **C:**  
1732 Analysis excluding the optic lobe neuropil. Species data points are indicated by the  
1733 first letter of their genus and species name: *D.p* = *Danaus plexippus*; *H.e* = *Heliconius*  
1734 *erato*; *H.h* = *H. hecale*; *G.z* = *Godyris zavaleta*; *H.v* = *Heliothis virescens*; *M.s* =  
1735 *Manduca sexta*. **D:** The proportion of the midbrain occupied by MB-ca (dark red) and  
1736 MB-lo+pe (light red) in four butterflies, and two other insects with fully comparable  
1737 data. **E:** Across a wider sample of insects (shown in A), when expressed as a  
1738 percentage of total volume of OL, AL, CB and MB, *Apis mellifera* (solid blue) and  
1739 *Heliconius* (solid red) stand out as having expanded mushroom bodies, correcting for

1740 the size of the optic neuropil, compared to other Lepidoptera (unfilled red circles) and  
1741 other insects (unfilled blue circles). The line was fitted by PGLS. All insect images in  
1742 A are from Wikimedia commons and were released under the Creative Commons  
1743 License, except *Heliconius* (see Fig. 1).

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1770 **Tables**

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1772 **Table 1:** Neuropil volumes and body size of A) *H. erato* and B) *H. hecale*

1773

1774 **Table 2:** Comparisons between old (O) and young (Y) insectary-reared individuals  
1775 for A) *H. erato* and B) *H. hecale*.  $r$  is the effect size. DI indicates the group with a  
1776 higher value of  $\alpha$ ,  $\beta$  or fitted axis mean.

1777

1778 **Table 3:** Comparisons between wild caught (W) and old insectary-reared individuals  
1779 for A) *H. erato* and B) *H. hecale*.  $r$  is the effect size. DI indicates the group with a  
1780 higher value of  $\alpha$ ,  $\beta$  or fitted axis mean.

1781

1782 **Table 4:** Comparisons between *H. erato* and *H. hecale* for A) wild caught and B) old  
1783 insectary-reared individuals.  $r$  is the effect size. DI indicates the species with a higher  
1784 value of  $\alpha$ ,  $\beta$  or fitted axis mean.

1785

1786 **Table 5:** Discriminant function analysis of variation between wild and old insectary-  
1787 reared *H. erato* and *H. hecale*. A) Canonical discriminant function coefficients for  
1788 DF1-3. B) Discriminant function statistics. C) Results of a MANOVA of DF1-3 to  
1789 test for associations with species, group, and species-group interactions.

1790

1791 **Table 6:** Loadings on Principal Components Analysis of the relative size of brain  
1792 components across six Lepidoptera.

1793

1794

**Table 1****A) *H. erato***

	<i>wild caught</i>				<i>old insectary reared</i>		<i>young insectary reared</i>	
	mean ( <i>n</i> = 10)	SD	Rel. SD (%)	% total Neuropil	mean ( <i>n</i> = 10)	SD	mean ( <i>n</i> = 10)	SD
Body mass (g)	0.093	0.017	19.999	-	0.074	0.014	0.088	0.019
Body length (mm)	23.833	1.426	5.983	-	23.095	1.773	22.671	0.951
Wing span (mm)	71.408	3.278	4.591	-	69.744	4.12	68.786	2.55
Lamina	7.409E+07	1.052E+07	14.192	13.459	6.95E+07	1.61E+07	5.49E+07	1.25E+07
Medulla	2.396E+08	3.617E+07	15.094	43.523	2.45E+08	2.76E+07	1.90E+08	3.32E+07
Accessory medulla	1.633E+05	3.609E+04	22.094	0.030	1.59E+05	4.61E+04	9.77E+04	1.93E+04
Inner lobula	2.630E+07	4.203E+06	15.984	4.777	2.79E+07	2.89E+06	2.07E+07	4.32E+06
Lobula plate	1.393E+07	2.083E+06	14.952	2.531	1.35E+07	2.22E+06	1.04E+07	2.07E+06
OG	1.054E+06	2.400E+05	22.769	0.191	1.05E+06	2.42E+05	8.85E+05	2.26E+05
Antennal lobes	1.185E+07	2.450E+06	20.671	2.153	1.19E+07	2.49E+06	7.72E+06	1.10E+06
AOTu	2.199E+06	4.535E+05	20.618	0.400	2.26E+06	3.28E+05	1.52E+06	3.27E+05
MB calyx	4.672E+07	9.290E+06	19.886	8.486	4.50E+07	1.22E+07	2.79E+07	5.75E+06
MB peduncle	6.043E+06	1.109E+06	18.343	1.098	6.15E+06	1.35E+06	5.57E+06	1.58E+06
MB lobes	2.267E+07	5.812E+06	25.641	4.118	2.17E+07	4.26E+06	1.28E+07	2.31E+06
Central body lower	3.017E+05	5.189E+04	17.198	0.055	2.83E+05	6.00E+04	2.24E+05	3.81E+04
Central body upper	1.180E+06	1.788E+05	15.153	0.214	1.17E+06	2.57E+05	8.90E+05	1.36E+05
Noduli	2.966E+04	1.146E+04	38.631	0.005	3.09E+04	1.64E+04	3.16E+04	8.46E+03
Protocerebral bridge	2.120E+05	4.804E+04	22.658	0.039	1.96E+05	5.04E+04	1.39E+05	2.02E+04
POTu	4.213E+04	9.976E+03	23.681	0.008	4.20E+04	1.43E+04	2.73E+04	7.93E+03
Total midbrain	1.954E+08	3.365E+07	17.222	35.490	2.04E+08	2.70E+07	1.39E+08	2.28E+07

**Table 1 continued**

**B) *H. hecale***

	<i>wild caught</i>				<i>old insectary reared</i>		<i>young insectary reared</i>	
	mean ( <i>n</i> = 10)	SD	Rel. SD (%)	% total Neuropil	mean ( <i>n</i> = 9)	SD	mean ( <i>n</i> = 10)	SD
Body mass (g)	0.163	0.025	15.317	-	0.154	0.046	0.171	0.047
Body length (mm)	29.693	3.097	10.431	-	28.189	3.0631	29.206	2.75
Wing span (mm)	88.129	8.004	9.082	-	80.6	7.134	86.34	8.012
Lamina	9.751E+07	1.826E+07	18.721	13.939	9.39E+07	2.17E+07	9.64E+07	1.50E+07
Medulla	2.986E+08	5.342E+07	17.888	42.689	2.48E+08	3.81E+07	2.42E+08	3.66E+07
Accessory medulla	1.660E+05	2.951E+04	17.782	0.024	1.40E+05	2.80E+04	1.38E+05	3.67E+04
Inner lobula	3.056E+07	5.630E+06	18.422	4.369	2.80E+07	4.64E+06	2.45E+07	5.06E+06
Lobula plate	1.648E+07	2.972E+06	18.031	2.356	1.45E+07	2.45E+06	1.27E+07	2.53E+06
OG	1.099E+06	3.396E+05	30.894	0.157	9.93E+05	2.12E+05	9.24E+05	2.10E+05
Antennal lobes	1.216E+07	2.056E+06	16.905	1.739	1.09E+07	1.34E+06	9.36E+06	1.59E+06
AOTu	2.572E+06	6.144E+05	23.891	0.368	2.30E+06	4.46E+05	2.02E+06	3.76E+05
MB calyx	5.271E+07	1.611E+07	30.569	7.534	3.60E+07	7.49E+06	2.60E+07	7.48E+06
MB peduncle	6.680E+06	1.525E+06	22.834	0.955	5.92E+06	1.30E+06	4.91E+06	1.39E+06
MB lobes	2.421E+07	6.279E+06	25.930	3.461	1.79E+07	3.56E+06	1.32E+07	3.51E+06
Central body lower	3.109E+05	6.362E+04	20.467	0.044	2.91E+05	7.15E+04	2.47E+05	3.74E+04
Central body upper	1.093E+06	2.026E+05	18.541	0.156	1.16E+06	2.05E+05	9.65E+05	1.79E+05
Noduli	4.207E+04	1.713E+04	40.730	0.006	3.34E+04	8.35E+03	3.06E+04	1.28E+04
Protocerebral bridge	2.424E+05	5.657E+04	23.335	0.035	2.00E+05	3.09E+04	1.64E+05	1.75E+04
POTu	4.183E+04	1.257E+04	30.057	0.006	3.74E+04	8.47E+03	3.20E+04	8.27E+03
Total midbrain	2.551E+08	6.253E+07	24.513	36.465	1.82E+08	2.28E+07	1.50E+08	2.25E+07















**Table 5**

**A) Canonical discriminant function coefficients**

<b>Neuropil</b>	<b>DF 1</b>	<b>DF 2</b>	<b>DF 3</b>
rMid	1.716	0.892	-1.404
aME	-0.177	-0.190	0.093
ME	0.167	2.615	0.356
Lo	-1.906	-0.122	-0.802
LoP	0.000	-2.417	-0.287
OG	-0.224	-0.451	0.412
La	1.626	-0.517	0.591
AOTu	-0.570	-0.232	0.369
AL	-1.117	-0.565	0.296
MB-ca	-0.467	0.238	1.950
MB-lo+pe	-0.074	0.353	-0.935
CB	0.860	-0.619	-0.777
PB	0.755	0.741	0.726
POTu	-0.311	0.472	0.161

**B) Discriminant function statistics**

Correct group assignment		87%	
Eignevalue	3.090	2.065	0.528
% of variance	54.400	36.300	9.300
Wilks' Lambda	0.052	0.214	0.655
$\chi^2$	70.855	37.047	10.169
p	0.004	0.074	0.601

**C) MANOVA statistics**

F1(Species)	97.2632***	0.550	1.491
F1(Group)	4.6411*	11.695***	15.546***
F1(Species*Group)	0.000	38.172***	1.184

**Table 6**

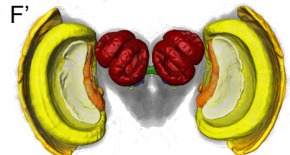
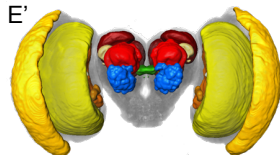
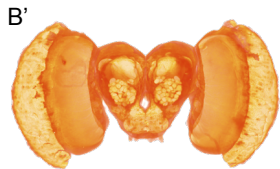
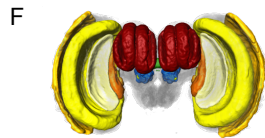
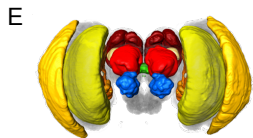
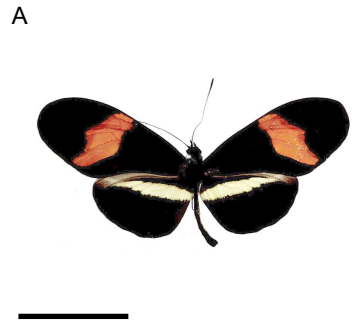
**A) Midbrain only**

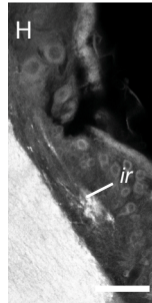
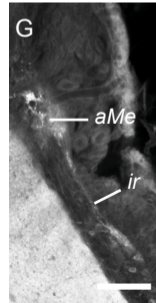
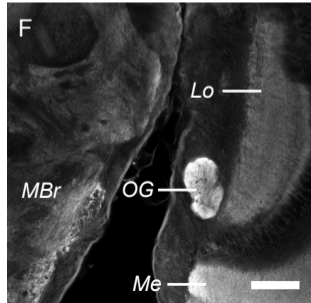
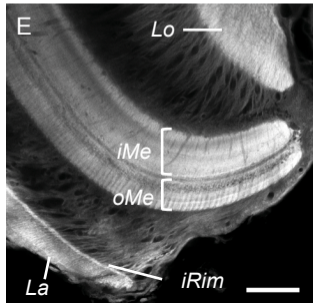
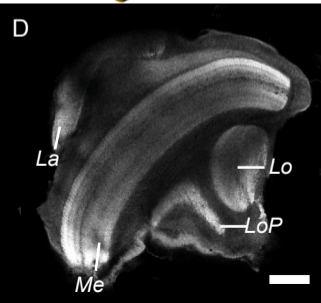
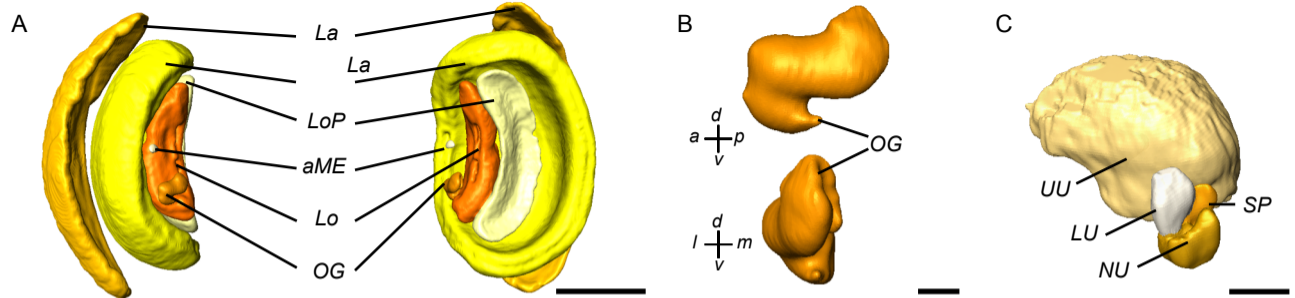
<b>Neuropil</b>	<b>Loadings</b>	
	<b>Residuals</b>	
	<b>PC1</b>	<b>PC2</b>
Antennal lobe	-0.981	-0.045
CB L+U	-0.798	0.406
MB Calyx	0.962	0.11
MB lobes+peduncle	0.952	0.231
AOTu	-0.047	0.966

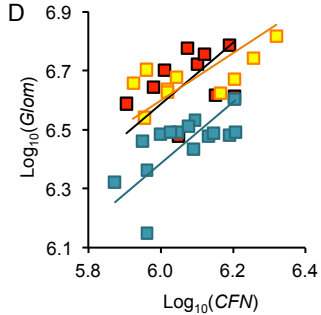
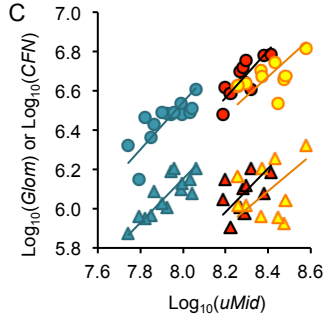
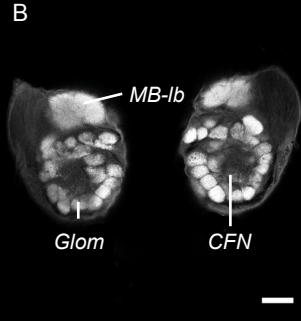
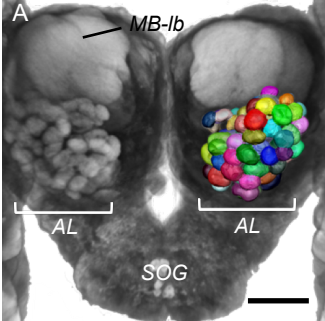
**B) Whole neuropil**

<b>Neuropil</b>	<b>Loadings</b>	
	<b>Residuals</b>	
	<b>PC1</b>	<b>PC2</b>
Antennal lobe	0.761	0.619
CB L+U	0.671	0.67
MB Calyx	-0.961	0.212
MB lobes+peduncle	-0.942	0.222
AOTu	0.811	0.024
Medulla	0.042	-0.949
Lobula	0.92	-0.354
Lobula plate	0.962	-0.167

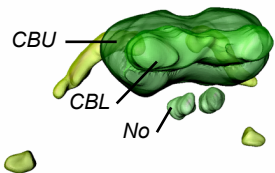




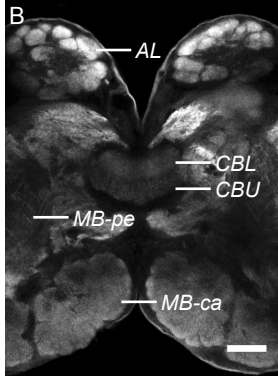




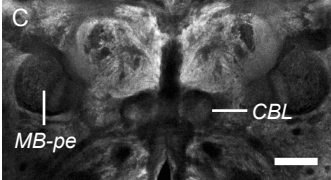
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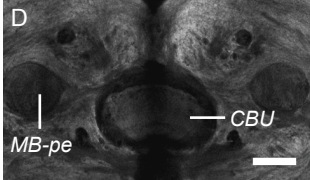
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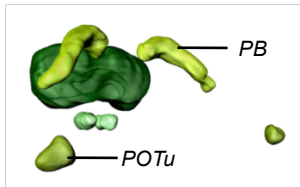
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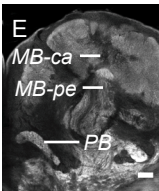
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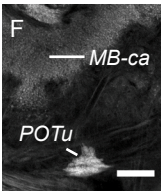
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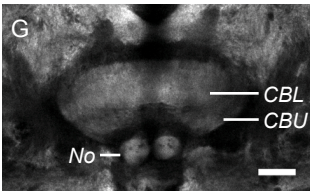
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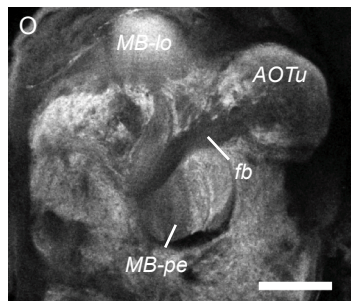
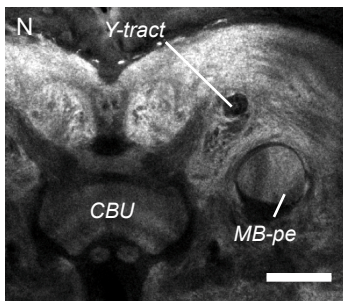
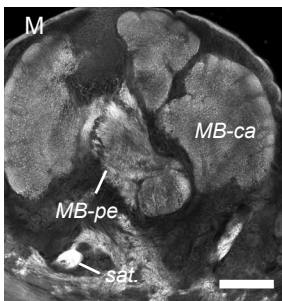
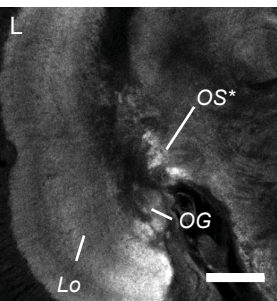
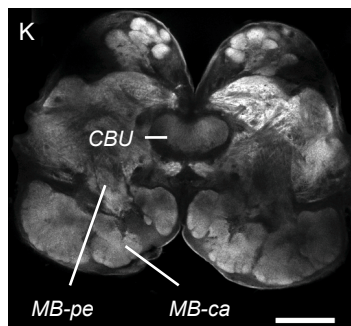
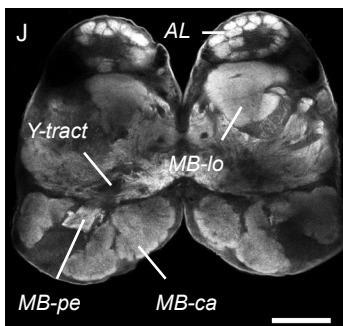
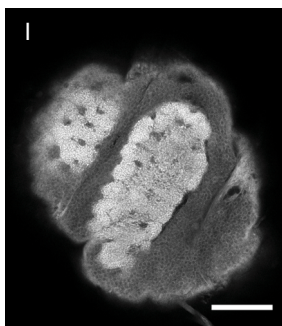
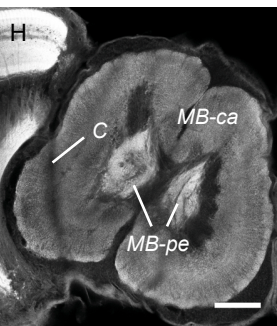
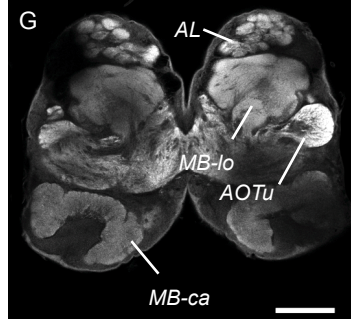
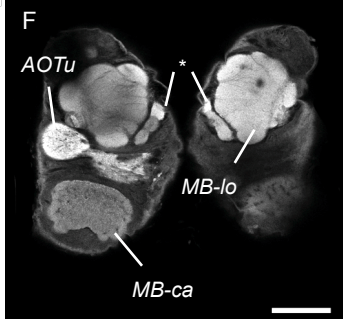
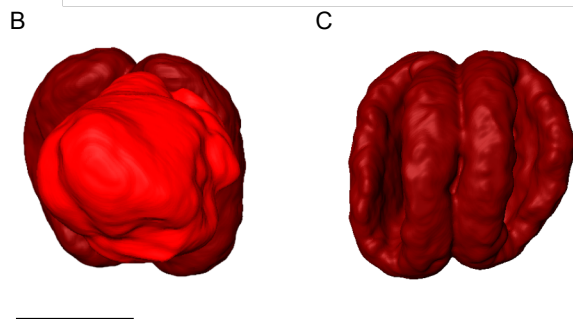
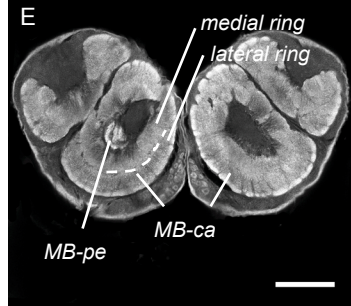
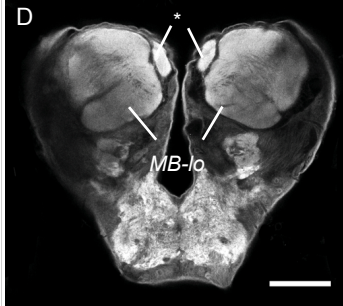
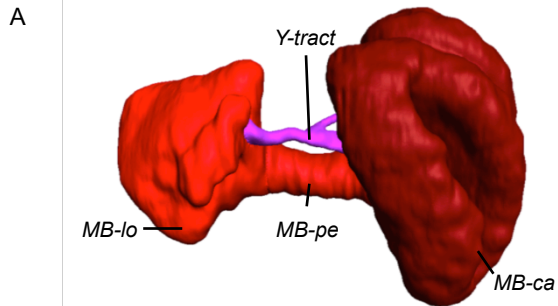


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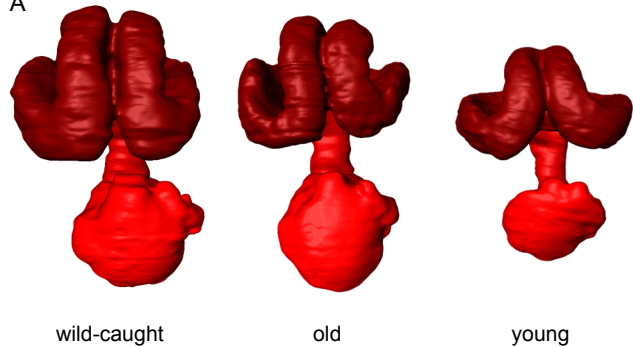


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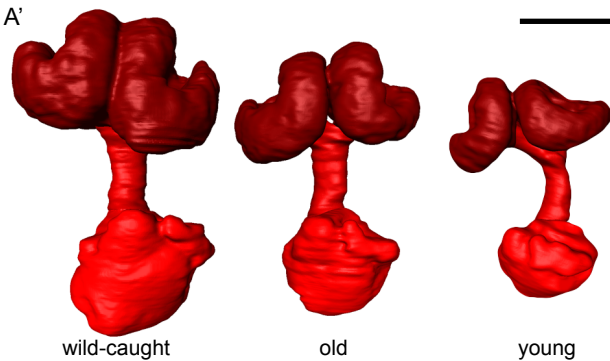




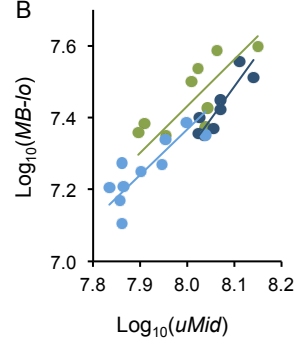
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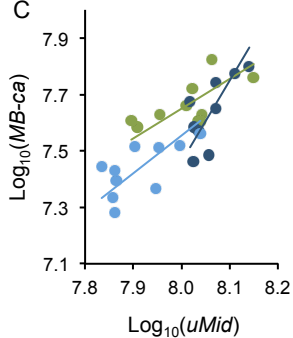
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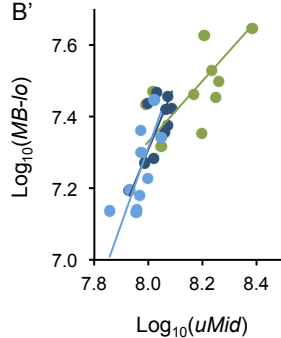
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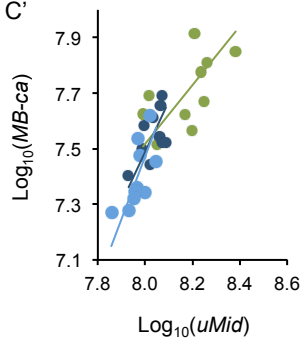
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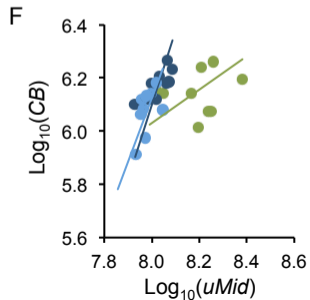
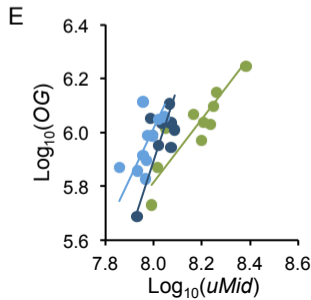
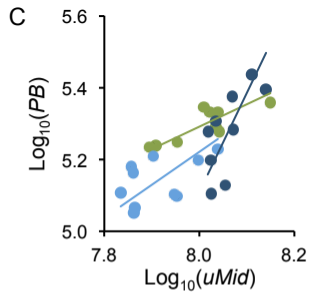
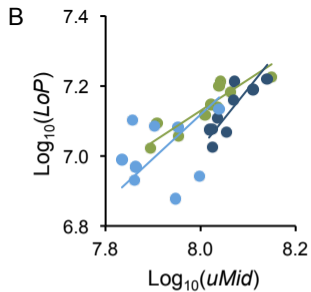
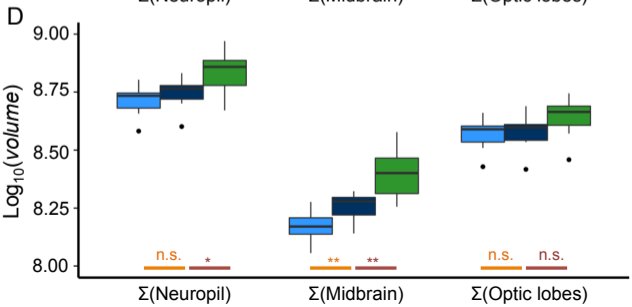
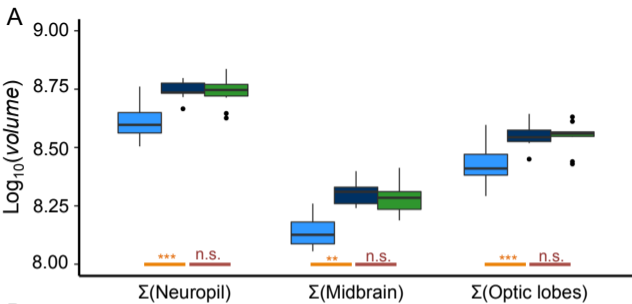


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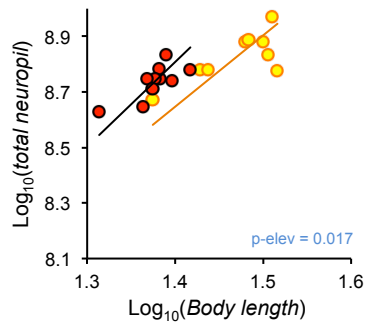


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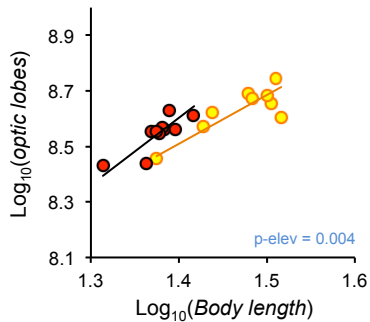




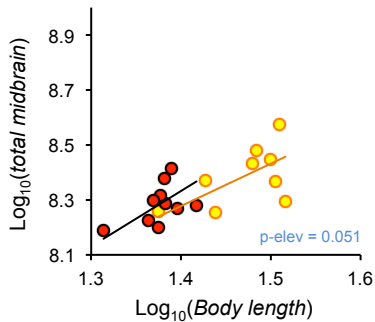
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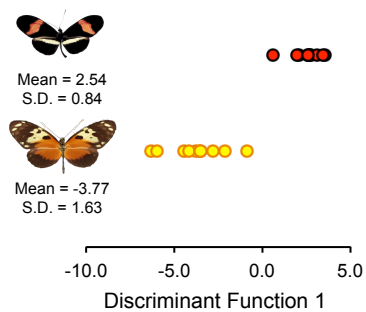
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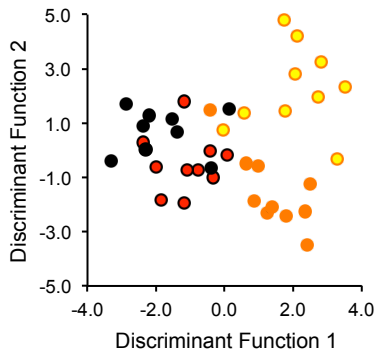
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