

20 **Abstract**

21 Sexually dimorphic behavior is pervasive across animals, with males and females
22 exhibiting different mate selection, parental care, foraging, dispersal, and territorial
23 strategies. However, the genetic underpinnings of sexually dimorphic behaviors are
24 poorly understood. Here we investigate gene networks and expression patterns associated
25 with sexually dimorphic imprinting-like learning in the butterfly *Bicyclus anynana*. In
26 this species, both males and females learn visual preferences, but learn preferences for
27 different traits and use different signals as salient, unconditioned cues. To identify genes
28 and gene networks associated with this behavior, we examined gene expression profiles
29 of the brains and eyes of male and female butterflies immediately post training and
30 compared them to the same tissues of naïve individuals. We found more differentially
31 expressed genes and a greater number of significant gene networks in the eye, indicating
32 a role of the peripheral nervous system in visual imprinting-like learning. Females had
33 higher chemoreceptor expression levels than males, supporting the hypothesized sexual
34 dimorphic use of chemical cues during the learning process. In addition, genes that
35 influence *B. anynana* wing patterns (sexual ornaments), such as *invected*, *spalt*, and
36 *apterous*, were also differentially expressed in the brain and eye, suggesting that these
37 genes may influence both sexual ornaments and the preferences for these ornaments. Our
38 results indicate dynamic and sex-specific responses to social scenario in both the
39 peripheral and central nervous systems and highlight the potential role of wing patterning
40 genes in mate preference and learning across the Lepidoptera.

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42 **Key words:** mate choice; sexual imprinting; butterfly; transcriptomics; wing patterning

43

44 **Introduction**

45 Sexually dimorphic behavior is pervasive across animal taxa. Males and females
46 may exhibit different mate selection strategies (Byrne and Rice, 2006; Kokko and
47 Johnstone, 2002; Talyn and Dowse, 2004), parental care behavior (Trivers, 1972; Zilkha
48 et al., 2017), foraging strategies (Ehl et al., 2018; Quillfeldt et al., 2011; Shannon et al.,
49 2006), dispersal (reviewed in (Greenwood, 1980; Trochet et al., 2016)), and territorial
50 displays (Reedy et al., 2017; Rosell and Thomsen, 2006). Though pervasive across
51 species and context, the genetic underpinnings of many types of sexually dimorphic
52 behavior are poorly understood. This is partially because males and females carry much
53 of the same genetic material; thus, sex-specific behavior is unlikely to be allele
54 dependent, except for the rare behaviors that are primarily associated with genes of large
55 effect on the sex chromosome. And, because behaviors are notoriously complex traits,
56 even sexually dimorphic behaviors influenced by genes of large effect on the sex
57 chromosome are likely to also be influenced by autosomal genes of minor effect
58 (Edwards et al., 2009; Lande, 1980).

59 Substantial headway has been made in elucidating the hormones and genes that
60 act as master regulators of sexually dimorphic traits and behaviors in model systems.
61 Sex-specific steroid hormone production is associated with sexually dimorphic behaviors
62 such as song production in song birds (Alward et al., 2013; Gurney and Konishi, 1980),
63 aggression in mammals (reviewed in (Hashikawa et al., 2018)), and spawning in fish
64 (Pradhan and Olsson, 2015). Similarly, sex-specific alternative splicing of master
65 regulator genes, such as *doublesex*, is associated with sexually dimorphic morphology

66 and behavior in arthropods (Kunte et al., 2014; Rideout et al., 2007; Rodriguez-Caro et
67 al., 2021; Wang et al., 2020). However, hormones and genes such as *doublesex* are often
68 upstream master regulators, and the presumably sexually dimorphic downstream gene
69 networks associated with hormone- and *doublesex*-related behaviors remain largely
70 unknown, outside of courtship initiation in the fruit fly *Drosophila melanogaster* (Datta
71 et al., 2008; Ruta et al., 2010) and song production in the zebra finch *Taeniopygia guttata*
72 (Olson et al., 2015; Woodgate et al., 2014) and the canary *Serinus canaria* (Alward et al.,
73 2018).

74 One sexually dimorphic behavior that is pervasive across animals is imprinting-
75 like mate preference learning. In imprinting-like mate preference learning, sexually
76 immature, or juvenile, individuals learn preferences for characteristics of adults (often,
77 but not always parents) of the opposite sex (Immelmann, 1975; ten Cate and Vos, 1999;
78 Verzijden et al., 2012). This behavior is inherently sexually dimorphic, as females learn
79 preferences for male traits, and males learn preferences for female traits (Kendrick et al.,
80 2001; ten Cate, 1985; Verzijden et al., 2008; Witte and Sawka, 2003). The sexual
81 dimorphism in trait learning can be quite extreme if adults are highly sexually dimorphic
82 or there are sex-specific signal modalities, such as male-limited pheromones or song.

83 To better understand the gene networks underlying sexual dimorphism in
84 imprinting-like learning, we examined sex-specific gene expression patterns in the brains
85 and eyes of *Bicyclus anynana* butterflies during an imprinting-like learning event. Both
86 male and female *B. anynana* butterflies exhibit imprinting-like learning, but they learn
87 preferences for different traits. Female *B. anynana* learn preferences for numbers of
88 dorsal forewing eyespots and are better at learning preferences for increasing numbers of

89 spots (Westerman et al., 2012). Conversely, male *B. anynana* learn preferences for dorsal
90 hindwing eyespots and are better at learning preferences for loss of spots (Westerman et
91 al., 2014). In addition to the observed sexual dimorphism in traits learned and
92 directionality of learning bias, females learn from males who exude a volatile sex
93 pheromone (Nieberding et al., 2008; Nieberding et al., 2012; Westerman and Monteiro,
94 2013), while males learn from females who, to our knowledge, do not have a volatile sex
95 pheromone. Thus, the two sexes are likely using different cues as unconditioned stimuli
96 to induce imprinting-like learning.

97 This sexual dimorphism in learning could be associated with sexual dimorphism
98 in perception, sexual dimorphism in downstream neural processing, or a combination of
99 these two processes. Previous studies suggest that male *B. anynana* have larger eyes and
100 more facets (ommatidia) than female *B. anynana*, and consequently, they potentially have
101 greater spatial acuity (Everett et al., 2012; Macias-Muñoz et al., 2015). If the observed
102 sexual dimorphism in learning is primarily associated with sexual dimorphism in visual
103 perception, we expect to see differential gene expression in the eyes of female and male
104 butterflies and in visual processing genes in the brain. Alternatively, the observed sexual
105 dimorphism in learning could be associated with sex-specific downstream processing, as
106 is seen in *D. melanogaster*'s response to pheromones (Datta et al., 2008; Ruta et al.,
107 2010). In this case we expect to find differential expression of genes unrelated to visual
108 processing in the brains of males and females. We might also find differential expression
109 of putative “magic genes,” genes subject to divergent selection that also pleiotropically
110 affect reproductive isolation, potentially by being associated with both the production of
111 and preference for given a trait (Servedio et al., 2011), such as butterfly wing patterning

112 genes. Many wing patterning genes are expressed in the heads of *B. anynana* (Ernst and
113 Westerman, 2021), and males and females have different wing patterns, with males
114 having brighter UV-reflective eyespots than females (Everett et al., 2012; Prudic et al.,
115 2011) while females have more dorsal hindwing spots than males (Westerman et al.,
116 2014). Additionally, since males but not females produce pheromones that can act as the
117 unconditioned stimuli for learning (Nieberding et al., 2008; Westerman and Monteiro,
118 2013), we may identify female-specific expression of genes in chemosensory processing
119 pathways.

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121 **Results**

122 To examine sex-specific gene expression in the brains and eyes of *Bicyclus*
123 *anynana* butterflies during an imprinting-like learning event, both male and female *B.*
124 *anynana* butterflies were either subjected to an imprinting-like learning event with a
125 conspecific of the opposite sex bearing modified wing ornaments or were placed in a
126 cage alone as a control (Fig. 1A). These two treatments mirror the experiences of trained
127 and naïve individuals prior to mate choice assays in published butterfly imprinting-like
128 learning studies (Westerman et al., 2012; Westerman and Monteiro, 2013; Westerman et
129 al., 2014). Butterflies were observed during these training/control periods, and the
130 corresponding behavioral data were analyzed to confirm that sex-specific expression
131 patterns were not the result of sexually dimorphic activity levels (S1 Table & S2 Table).
132 We then sequenced the eye and brain transcriptomes of these animals, N=10 per
133 treatment per sex, which generated a total of nearly three billion high-quality 50 base pair
134 (bp) single end (SE) reads (S3 Table). Approximately 1.6 million reads (0.05% of raw

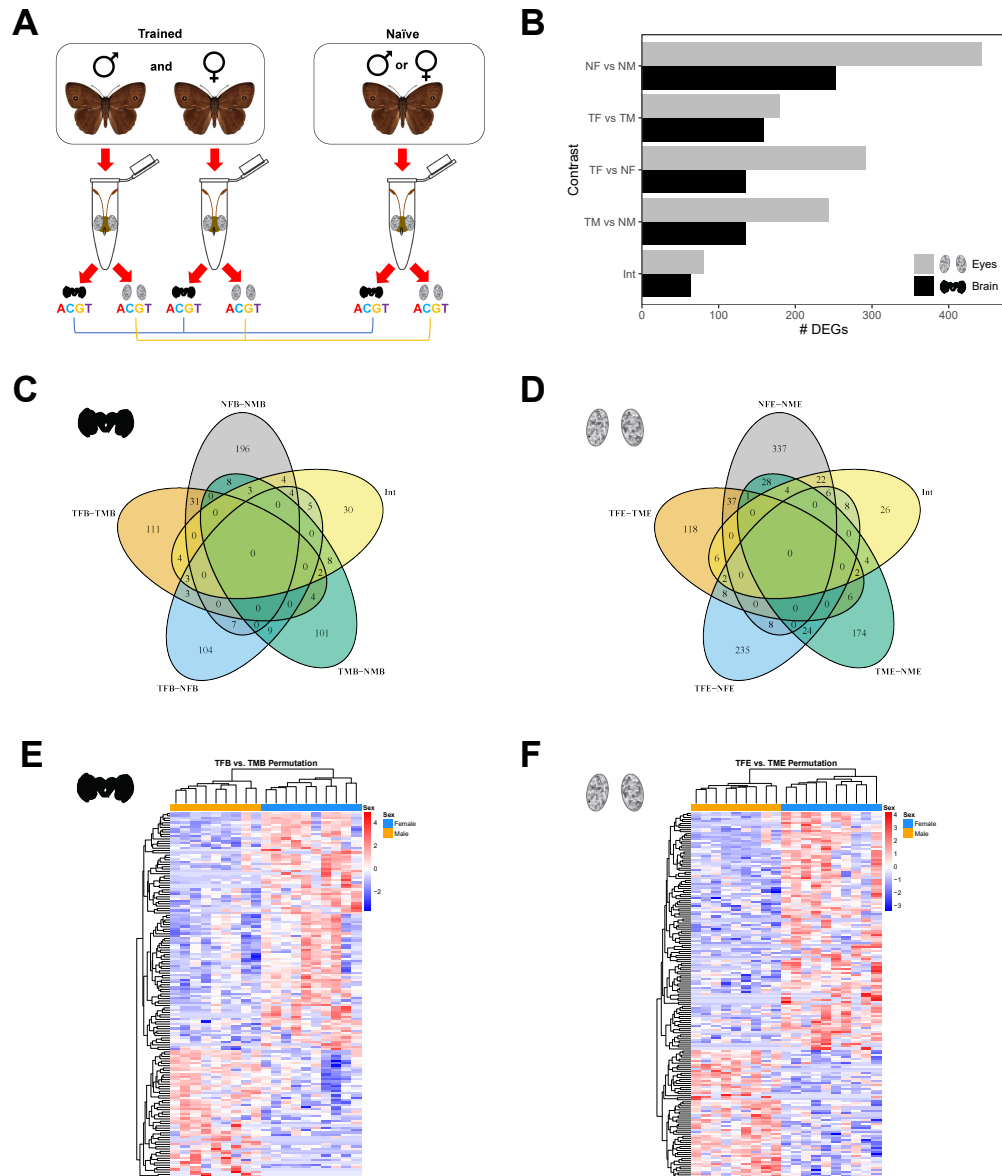
135 reads) were removed during adapter trimming, with 2.7 billion of the remaining reads
136 (90% of trimmed reads) mapping to the *B. anynana* reference genome (Nowell et al.,
137 2017). Across all brain libraries, 16,785 genes (74% of annotated genes in the genome)
138 had at least 10 mapped reads, while this was the case for 16,612 genes (73%) for eye
139 libraries. For each tissue, these gene sets were used as input for differential expression
140 analyses.

141 During data quality assessment, gene expression clustering analysis revealed that
142 one sample (TMB_E2, a trained male brain sample) was likely mislabeled, as it clustered
143 with eye samples (S1 Fig.). Because the two tissue types exhibited distinct clustering
144 patterns and tissue type accounted for approximately 85% of the variance, this sample
145 was discarded and not included in downstream analyses.

146 For all differential gene expression comparisons, we used DESeq2 to perform
147 both a standard differential expression analysis as well as a permutation-test-based
148 analysis, a method that eliminates the assumption of gene independence and provides a
149 more accurate representation of the data structure of gene expression datasets (Bloch et
150 al., 2018; Ghalambor et al., 2015; Slonim, 2002). Nearly all genes that were determined
151 to be differentially expressed in the standard DESeq2 analyses (Tables S4-S15) were also
152 identified as differentially expressed when employing permutation test analyses (Tables
153 S4-S15). Moreover, because the permutation test analyses reduce potential over-
154 correction by multiple testing correction methods, a larger number of differentially
155 expressed genes (DEGs) was found for all comparisons. Therefore, all downstream
156 analyses were conducted with the results of the permutation-based differential expression
157 tests. While all DEG sets obtained from these analyses were tested for gene ontology

158 (GO) term enrichment, GO term enrichment results are only reported for DEG sets with
 159 significantly enriched GO terms.

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164 **Figure 1: Experimental design and broadscale sexually dimorphic gene expression. A)**

165 Protocol for butterfly training and sampling. Newly emerged males/females were either solo or

166 paired with a two-day-old, zero-spot female/four-spot male. Heads of each focal animal were

167 collected, the brain and eyes dissected, and mRNA sequenced for expression analysis. B)

168 Numbers of differentially expressed genes for each comparison for each tissue. C) Brain Venn

169 diagrams showing overlap patterns for differentially expressed genes. D) Eye Venn diagrams
170 showing overlap patterns for differentially expressed genes. E) Brain gene expression heatmaps
171 of differentially expressed genes from trained females vs. trained males. Each row indicates a
172 single gene, and each column indicates an individual sample. Counts were first normalized by
173 variance stabilizing transformation, and gene-wise Z-scores were calculated for plotting. Genes
174 and samples are clustered by expression, with warmer colors denoting increased expression
175 relative to the mean for a given gene, while cooler colors denote decreased expression relative to
176 the mean. F) Eye gene expression heatmaps of differentially expressed genes from trained
177 females vs. trained males. NFB=naïve female brain, NMB=naïve male brain, TFB=trained female
178 brain, TMB=trained male brain, NFE=naïve female eye, NME=naïve male eye, TFE=trained
179 female eye, TME=trained male eye, Int=interaction.

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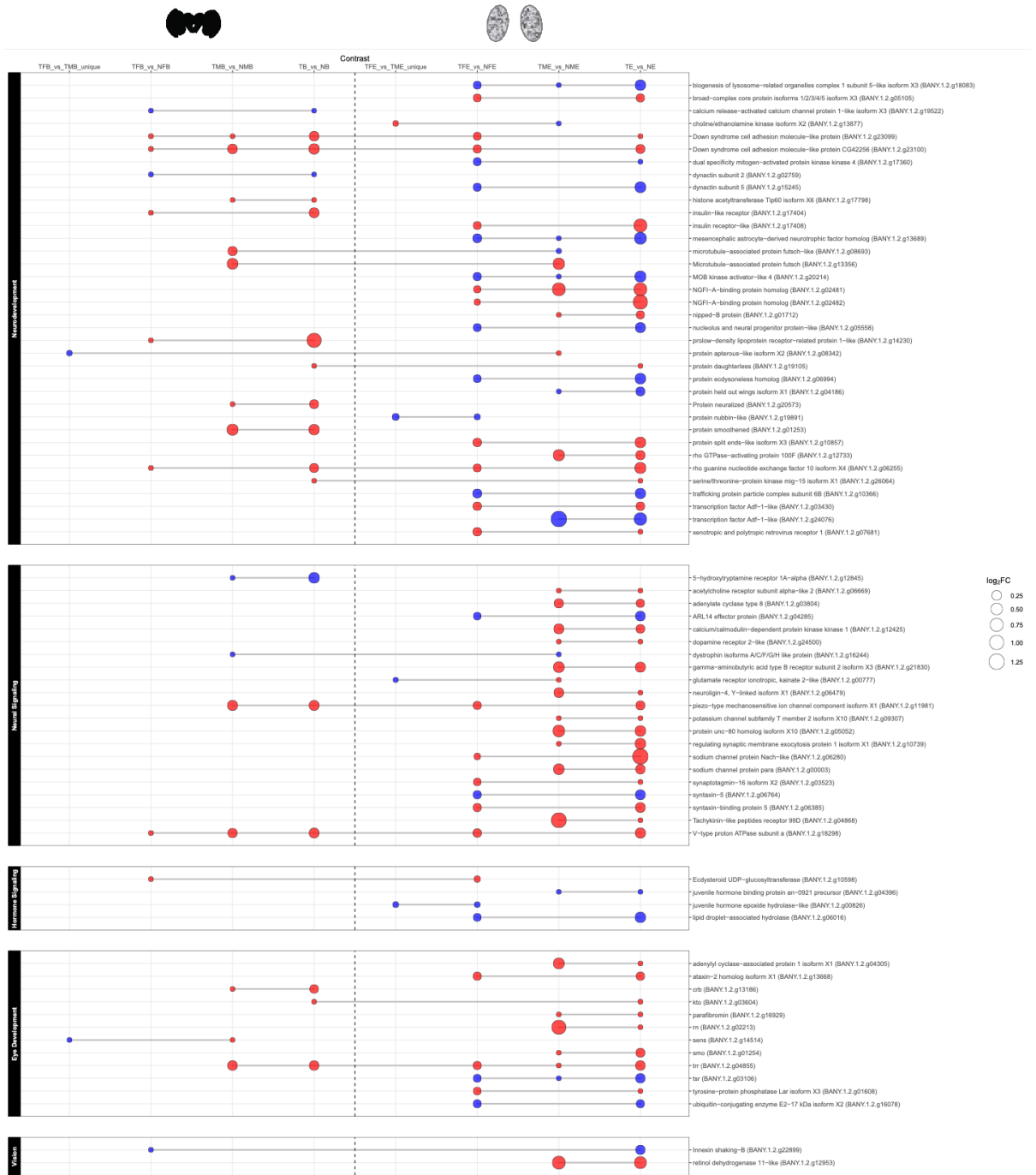
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182 *Trained male and female brains have distinct expression patterns*

183 Contrasting naïve female and male brains revealed a baseline of 253 genes that
184 were differentially expressed (Fig. 1B,C; S4 Table). Conversely, 158 genes were found to
185 be differentially expressed between trained female and male brains (Fig. 1B,C,E; S5
186 Table). Of these gene sets, 127 genes were unique to the training contrast (Fig. 1C),
187 several of which are linked to various neural processes, including neurodevelopment,
188 neural signaling, eye development, and phototransduction (Fig. 2; S16 Table).
189 Additionally, four genes with putative chemosensory functions were differentially
190 expressed, all of which were upregulated in females relative to males (chemosensory
191 protein 6, *BANY.1.2.g12995*; ejaculatory bulb-specific protein 3-like, *BANY.1.2.g12992*;
192 ejaculatory bulb-specific protein 3-like, *BANY.1.2.g12993*; and odorant receptor Or2-like,
193 *BANY.1.2.g25738*) (Ernst and Westerman, 2021). Finally, a gene encoding vitellogenin-
194 like (*BANY.1.2.g11921*), a protein known to influence the social behavior of numerous
195 insect species (Morandin et al., 2019; Nelson et al., 2007; Roy-Zokan et al., 2015), was
196 also upregulated in females.

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207 **Figure 2: Neural processing, hormone signaling, and vision genes are differentially**
208 **expressed in multiple contrasts.** The size of each dot indicates the effect size (\log_2FC), while
209 the color indicates the gene regulation relative to the first sample type listed for the contrast (e.g.,
210 for the TB vs. NB contrast, red indicates upregulation in trained brains, and blue indicates
211 downregulation in trained brains). Gray lines connecting the dots denote that the gene was
212 differentially expressed across multiple contrasts. NFB=naïve female brain, NMB=naïve male
213 brain, TFB=trained female brain, TMB=trained male brain, NFE=naïve female eye, NME=naïve
214 male eye, TFE=trained female eye, TME=trained male eye, TB=trained brain, TE=trained eye,
215 NB=naïve brain, NE=naïve eye.
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217 GOExpress analyses, which find gene ontology (GO) terms that best classify
218 samples from two separate groups, identified 171 GO terms that were significantly
219 associated with differences between naïve female and male brains ($p < 0.05$; S17 Table),
220 while 166 GO terms differentiated trained female and male brains ($p < 0.05$; S18 Table).
221 To eliminate baseline differences, we removed significant terms that were also found in
222 the naïve results, resulting in 51 GO terms linked to differences specific to training (S18
223 Table). Of these terms, several are linked to neural processing, including calmodulin
224 binding ($p = 0.004$), vesicle docking involved in exocytosis ($p = 0.042$), gap junction ($p =$
225 0.046), and neuropeptide signaling pathway ($p = 0.008$).

226

227 *Trained male and female eyes have distinct expression patterns*

228 Differential expression analysis for naïve female and male eyes found a baseline
229 of 443 genes that were differentially expressed between naïve female and male eyes (Fig.
230 1B,D; S6 Table). By contrast, 180 DEGs were found for the trained female vs. male
231 comparison (Fig. 1B,D,F; S7 Table). In total, 142 genes were unique to the trained eye
232 contrast (Fig. 1D), including genes encoding proteins linked to neurodevelopment, neural
233 signaling, hormone signaling, and vision (Fig. 2; S16 Table). Moreover, three genes
234 putatively linked to circadian rhythms showed differential expression, including circadian

235 clock-controlled protein-like (*BANY.1.2.g04378*), which was upregulated in males, and
236 circadian clock-controlled protein-like (*BANY.1.2.g05915*) and protein takeout-like
237 (*BANY.1.2.g05914*), which were both upregulated in females. The takeout gene (*to*) is
238 also associated with male courtship behavior in *D. melanogaster* (Dauwalder et al.,
239 2002).

240 GOExpress analyses revealed 165 and 138 GO terms that were significantly
241 linked to expression differences between the sexes for naïve and trained eyes,
242 respectively ($p < 0.05$; S19, S20 Tables). Removal of terms that overlapped both the
243 naïve and trained sets resulted in 37 GO terms linked to sex-specific differences in
244 response to training (S20 Table). A number of these terms were associated with neural
245 processes and sensory transduction, including chloride transmembrane transport ($p =$
246 0.007), chloride channel activity ($p = 0.01$), vesicle docking involved in exocytosis ($p =$
247 0.017), and G protein-coupled peptide receptor activity ($p = 0.025$).

248

249 *Training has sex-dependent effects on expression patterns in brains and eyes*

250 Sex-specific pairwise comparisons between trained and naïve tissues revealed
251 many DEGs in all sex-dependent comparisons.

252 Starting with the female comparisons, a total of 135 genes were found to be
253 differentially expressed between trained and naïve female brains (Fig. 1B,C; S8 Table),
254 many of which have potential roles in neural development, neural signaling, hormone
255 metabolism, and eye-related processes (Fig. 2; S16 Table).

256 For the trained vs. naïve female eyes comparison, differential expression analysis
257 identified 291 DEGs (Fig. 1B,D; S9 Table). GO enrichment analysis found 12 GO terms

258 enriched in this gene set, with the top being mitochondrion (FDR=4.04E-04),
259 intracellular organelle (FDR=4.04E-04), and organelle (FDR=5.23E-04) (S21 Table).
260 There were several genes of interest in the trained vs. naïve female eye contrast,
261 including genes linked to neural development and signaling, hormone signaling, eye
262 development, and vision (Fig. 2; S16 Table).

263 Similar to the female brains comparison, the trained vs. naïve male brains
264 comparison found 135 DEGs (Fig. 1B,C; S10 Table), including several genes associated
265 with neurodevelopment, neural signaling, and eye development (Fig. 2; S16 Table).

266 Differential expression analysis revealed 243 DEGs for the trained vs. naïve male
267 eyes comparison (Fig. 1B,D; S11 Table). Again, numerous genes involved with neural
268 development, neural signaling, hormone signaling, vision, and eye development were
269 found to be differentially expressed between trained and naïve male eyes (Fig. 2; S16
270 Table).

271 Moreover, 63 genes in the brain and 80 genes in the eye were found to have a
272 significant sex:condition interaction, indicating that training differentially affected their
273 expression in females versus males (Fig. 1B,C,D; S12, S13 Tables). In both tissues, these
274 sex:condition interactions were found for genes involved with neural development and
275 signaling, and interactions were also found for genes linked to eye development in the
276 eye comparison (S16 Table). In addition, a gene putatively involved with chemoreception
277 (olfactory receptor 21, *BANY.1.2.g12009*; (Ernst and Westerman, 2021)) and a gene
278 associated with regulating circadian rhythms (protein LSM12 homolog,
279 *BANY.1.2.g13734*; (Lee et al., 2017)) showed significant sex:condition interactions in the
280 brain and eyes, respectively.

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282 *Training has a sex-independent effect on gene expression in brains*

283 Testing for the overall effect of training while controlling for differences in
284 expression due to sex revealed 283 genes that were differentially expressed in trained vs.
285 naïve brains (Fig. S2A; S14 Table). Many of the genes in this gene set have functions
286 related to neurodevelopment, neural signaling, hormone signaling, and eye development
287 (Fig. 2; S16 Table). Moreover, LSM12 homolog (*BANY.1.2.g13734*), which showed
288 significant sex:condition interactions in the eyes, was also differentially expressed and
289 was upregulated in naïve brains.

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291 *Training has a sex-independent effect on gene expression in eyes*

292 In total, 658 DEGs were identified for the trained vs. naïve eyes comparison when
293 controlling for sex (Fig. S2B; S15 Table). GO enrichment analysis revealed 30 enriched
294 GO terms, with the top terms being mitochondrion (FDR=1.92E-06), protein-containing
295 complex (FDR=3.03E-06), and intracellular organelle (FDR=5.17E-06) (S22 Table).

296 Several of these DEGs have putative functions in neurodevelopment, neural
297 signaling, hormone signaling, eye development, and vision (Fig. 2; S16 Table). In
298 addition, a number of genes linked to learning and memory were differentially expressed
299 between trained and naïve eyes. Several of these genes were upregulated in trained eyes,
300 including nipped-B protein (*BANY.1.2.g01712*), Ca(2+)/calmodulin-responsive adenylate
301 cyclase (*BANY.1.2.g01825*), two transcription factor Adf-1-like (*BANY.1.2.g03430* and
302 *BANY.1.2.g08959*), adenylate cyclase type 8 (*BANY.1.2.g03804*), neurobeachin-like
303 (*BANY.1.2.g12252* and *BANY.1.2.g12258*), and ataxin-2 homolog isoform X1

304 (*BANY.1.2.g13668*). Conversely, cyclic AMP response element-binding protein B
305 isoform X3 (*BANY.1.2.g01685*), one transcription factor Adf-1-like (*BANY.1.2.g24076*),
306 probable RNA helicase armi isoform X1 (*BANY.1.2.g17424*), and fatty acid-binding
307 protein-like (*BANY.1.2.g17524*) were upregulated in naïve eyes. Finally, two genes
308 involved with male courtship in *Drosophila* (calcium/calmodulin-dependent 3',5'-cyclic
309 nucleotide phosphodiesterase 1 isoform X1, *BANY.1.2.g07806*; and cytoplasmic dynein 2
310 heavy chain 1, *BANY.1.2.g19627*) were upregulated in *B. anynana* eyes in the training
311 condition.

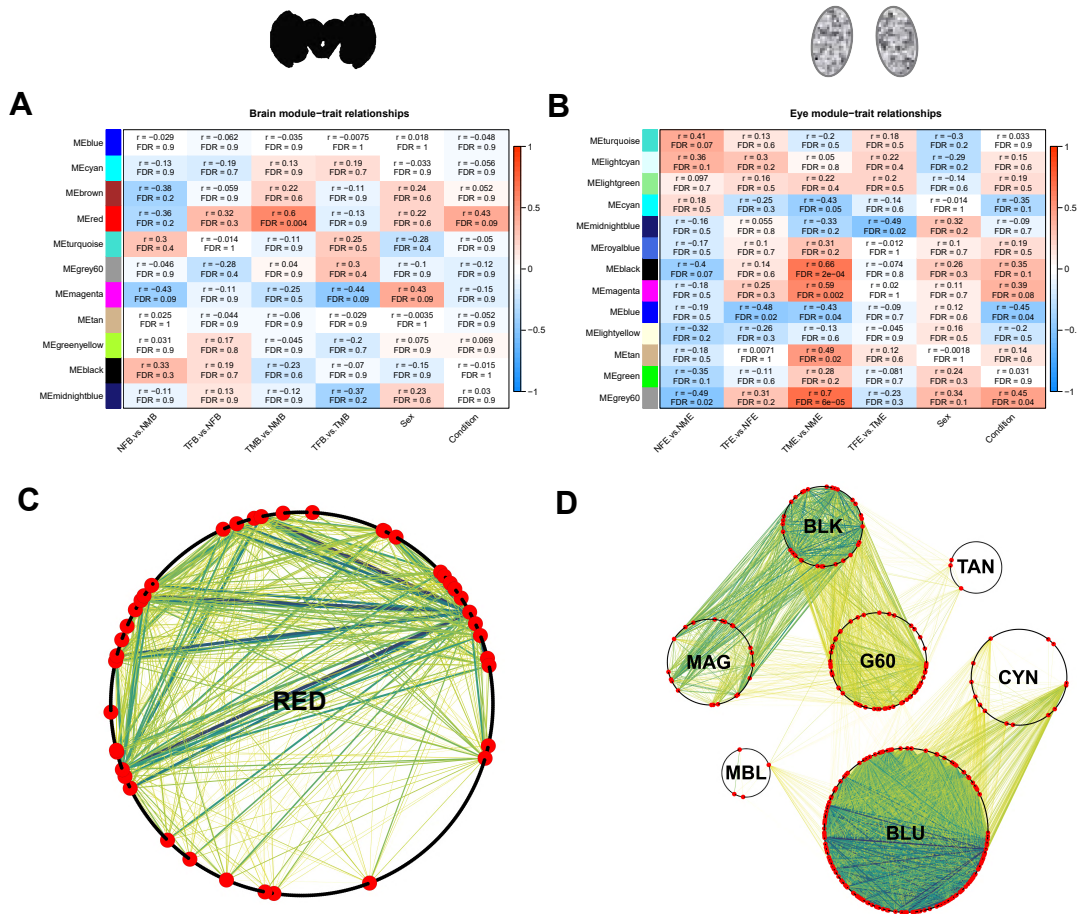
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313 *One gene network is associated with training condition in the brain*

314 To investigate gene networks that are associated with an imprinting-like learning
315 experience, we performed tissue-specific weighted gene co-expression network analyses
316 (WGCNA). Brain co-expression network analysis identified 17 modules, which was
317 reduced to 11 modules after merging highly correlated modules (Fig. 3A; S3A Fig.). Of
318 these modules, only one (the red module) was significantly correlated with a trait,
319 specifically the trained male brain vs. naïve male brain contrast (i.e., the red module was
320 significantly correlated with training condition for male brains; $r=0.6$; $FDR=0.004$) (Fig.
321 3C; S3B Fig.). This module consisted of 655 genes (S23 Table), with the top hub gene
322 (i.e., the most highly connected gene) identified as NADH dehydrogenase [ubiquinone] 1
323 beta subcomplex subunit 7-like (*BANY.1.2.g00209*). GO enrichment analyses found five
324 significantly enriched GO terms in the red module, which were linked to nucleic acid and
325 cyclic compound binding and mRNA metabolism (S24 Table; Fig. 4A).

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331 **Figure 3: Gene network modules in brain and eyes are significantly associated with**

332 **training.** Significant modules from co-expression analyses. A) Brain module-trait association

333 heatmap. Rows indicate module eigengenes (ME), and columns indicate the pairwise binary

334 indicators representing the various comparisons (“traits”) of interest. The top numbers in each cell

335 denote the correlation value (r), with false discovery rate (FDR) values below. Cells are colored

336 by the strength of the association, with r ranging from -1 to 1. B) Eye module-trait association

337 heatmap. C) WGCNA brain analysis red module Cytoscape plot. Each black dot around the

338 perimeter of the circle indicates a node (gene), with larger red dots indicating differentially

339 expressed genes from the contrast for which the module is significantly associated (i.e., trained

340 vs. naïve male brain). Each line indicates an edge (connection) for differentially expressed genes

341 within the module, with thinner yellow lines indicating weaker connections and thicker blue lines

342 indicating stronger connections. D) WGCNA eye analysis, Cytoscape plot of all significant

343 modules. Only edges for differentially expressed genes within and between modules are shown.

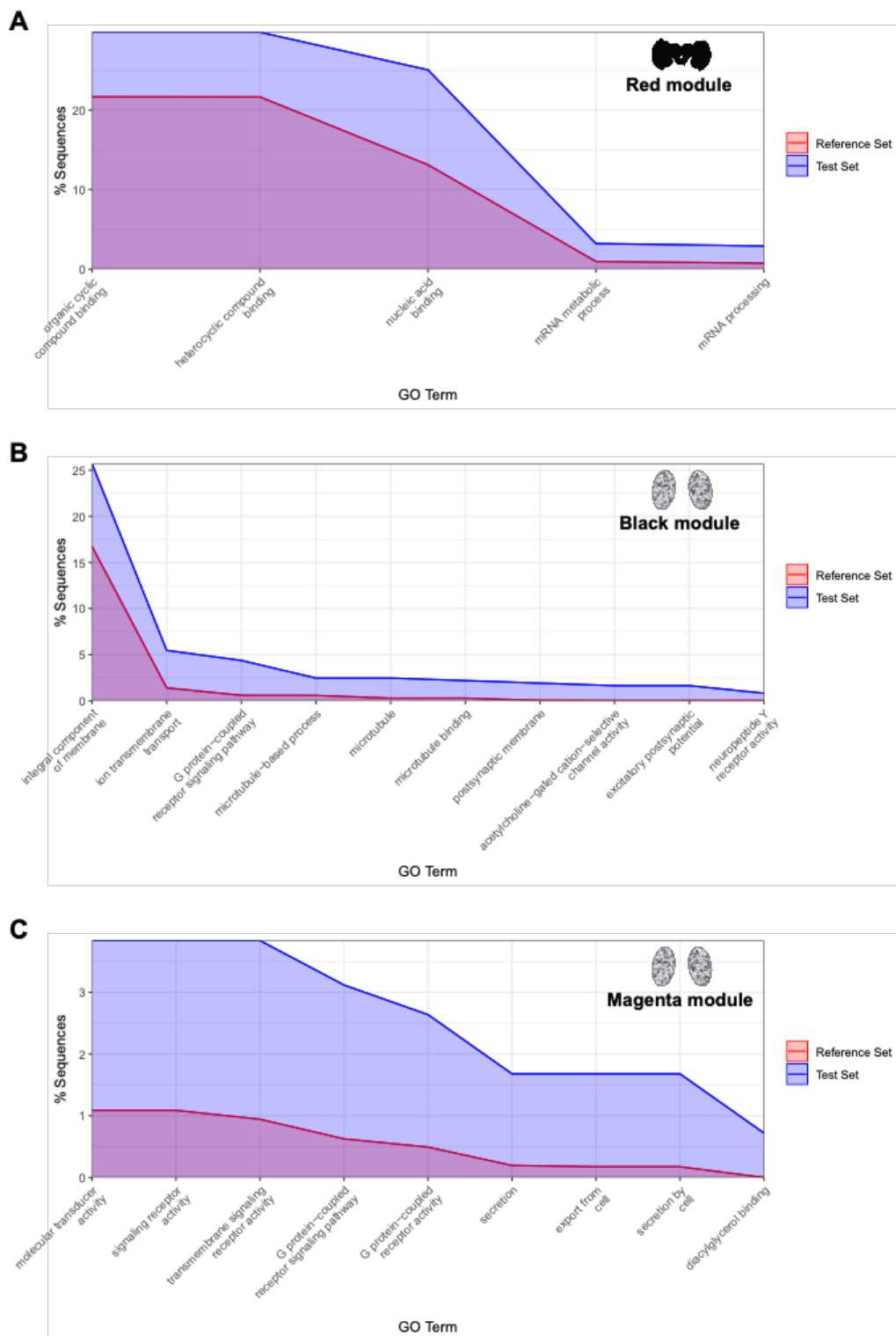
344 BLK=black module, BLU=blue module, CYN=cyan module, G60=grey60 module,

345 MAG=magenta module, MBL=midnightblue module, RED=red module, and TAN=tan module.

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351 **Figure 4: Gene ontology enrichment plots for significant brain and eye modules of interest.**

352 A) Significantly enriched GO terms in the brain red module. For each GO term, the percentage of

353 sequences annotated with that term within the Test Set (i.e., all red module genes) is plotted along

354 with the percentage of sequences annotated with that term within the Reference Set (i.e., all genes
355 used in the co-expression analysis). B) Significantly enriched GO terms in the eye black module.
356 Due to the large number of enriched GO terms in this module, only the most specific terms
357 identified by Blast2GO were plotted for clarity. C) Significantly enriched GO terms in the eye
358 magenta module.

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360 Many genes within the red module are linked to various neural and sensory
361 processes. Of particular interest, 41 DEGs identified in the trained vs. naïve male brain
362 contrast were also present in the red module network (S23 Table). Many of these genes
363 encode proteins linked to neural development, such as protein smoothened
364 (*BANY.1.2.g01253*), protein abrupt-like isoform X5 (*BANY.1.2.g17381*), histone
365 acetyltransferase Tip60 isoform X6 (*BANY.1.2.g17798*), Down syndrome cell adhesion
366 molecule-like protein (*BANY.1.2.g23099*), Down syndrome cell adhesion molecule-like
367 protein CG42256 (*BANY.1.2.g23100*), and helicase domino (*BANY.1.2.g24509*).
368 Additionally, others encode proteins involved with neural signaling, such as piezo-type
369 mechanosensitive ion channel component isoform X1 (*BANY.1.2.g11981*) and V-type
370 proton ATPase subunit a (*BANY.1.2.g18298*) and eye development, such as *trr*,
371 (*BANY.1.2.g04855*) and *crb* (*BANY.1.2.g13186*) (S16 Table). In addition to its role in eye
372 development, *trr* is also involved with short term courtship memory in *D. melanogaster*
373 (Sedkov et al., 2003).

374

375 *Several gene networks are associated with training condition in the eyes*

376 Eye co-expression network analysis identified 20 modules, which was reduced to
377 13 modules after merging highly correlated modules (Fig. 3B, S4A Fig.). Of these
378 modules, seven (the black, blue, cyan, grey60, magenta, midnight blue, and tan modules)

379 were significantly correlated with at least one contrast, and DEGs for the correlated
380 contrast(s) were present in all seven of these modules (Fig. 3D, S4 Fig.; Tables S25-S37).

381 Two of these modules (the black and magenta modules), both of which were
382 significantly correlated with the trained male vs. naïve male eyes contrast, were of
383 particular interest based on their GO enrichment profiles. The black module ($r=0.66$;
384 $FDR=2E-04$) consisted of 366 genes centered around the top hub gene gamma-
385 aminobutyric acid type B receptor subunit 2 (*BANY.1.2.g00039*), a component of the
386 receptor for the neurotransmitter GABA (S27 Table; Fig. 3D, S4C Fig.). Moreover, 73
387 GO terms were enriched in this module, most of which are associated with neural
388 processes (e.g., neurotransmitter receptor activity involved in regulation of postsynaptic
389 membrane potential, chemical synaptic transmission, and excitatory postsynaptic
390 potential) (S28 Table; Fig. 4B). A total of 32 DEGs from the trained male vs. naïve male
391 eyes contrast were found in the black module, nearly half of which are associated with
392 neural and eye development and neural signaling. Differentially expressed development
393 genes include protein unc-80 homolog isoform X10 (*BANY.1.2.g05052*), microtubule-
394 associated protein futsch-like (*BANY.1.2.g08693*), delta and Notch-like epidermal growth
395 factor-related receptor (*BANY.1.2.g09881*), protein abrupt-like isoform X1
396 (*BANY.1.2.g17383*), and *rst* (*BANY.1.2.g15359*) (S16 Table; S27 Table). Moreover,
397 differentially expressed neural signaling genes include sodium channel protein para
398 (*BANY.1.2.g00003*), potassium voltage-gated channel subfamily KQT member 1 isoform
399 X2 (*BANY.1.2.g01557*), adenylate cyclase type 8 (*BANY.1.2.g03804*), neuroligin-4, Y-
400 linked isoform X1 (*BANY.1.2.g06479*), acetylcholine receptor subunit alpha-like 2
401 (*BANY.1.2.g06669*), potassium channel subfamily T member 2 isoform X10

402 (*BANY.1.2.g09307*), calcium/calmodulin-dependent protein kinase kinase 1
403 (*BANY.1.2.g12425*), sodium leak channel non-selective protein (*BANY.1.2.g19402*),
404 gamma-aminobutyric acid type B receptor subunit 2 isoform X3 (*BANY.1.2.g21830*), and
405 dopamine receptor 2-like, (*BANY.1.2.g24500*) (S16 Table; S27 Table).

406 The magenta module ($r=0.59$; $FDR=0.002$) consisted of 417 genes with a hub
407 gene of disintegrin and metalloproteinase domain-containing protein 33-like (S29 Table;
408 S4D Fig.) and showed an enriched GO term profile similar to that of the black module
409 (S30 Table; Fig 4C). Specifically, the terms transmembrane signaling receptor activity, G
410 protein-coupled receptor signaling pathway, G protein-coupled receptor activity,
411 signaling receptor activity, and molecular transducer activity were found to be enriched
412 in both the black and magenta modules. In total, 21 DEGs from the trained male vs. naïve
413 male eyes contrast were found in the magenta module, a third of which have putative
414 functions in neurodevelopment (protein smoothed isoform X2, *BANY.1.2.g01254*;
415 putative defective proboscis extension response, *BANY.1.2.g12002*; rho GTPase-
416 activating protein 100F, *BANY.1.2.g12733*; and dynamin-like 120 kDa protein,
417 mitochondrial, *BANY.1.2.g23042*), neural signaling (regulating synaptic membrane
418 exocytosis protein 1 isoform X1, *BANY.1.2.g10739*; and dopamine receptor 1,
419 *BANY.1.2.g24271*), and eye development (adenylyl cyclase-associated protein 1 isoform
420 X1, *BANY.1.2.g04305*) (S16, S29 Tables).

421

422 *Wing patterning genes are differentially expressed in both the brain and eyes*

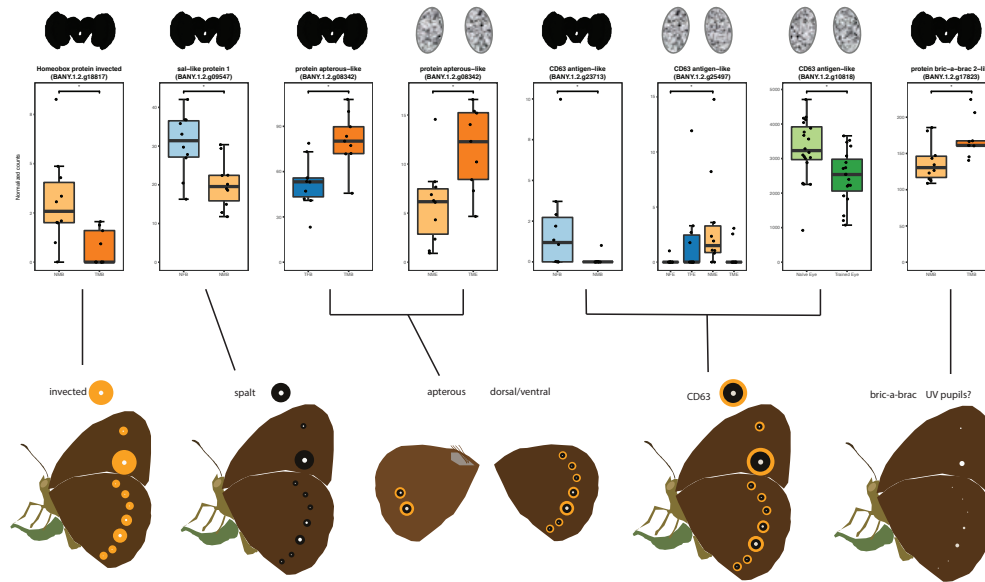
423 To investigate whether putative “magic genes,” or genes that influence both a
424 given trait as well as preference for that trait, are expressed in the brain and eyes of *B.*

425 *anymana*, we also explored the expression patterns of known butterfly wing patterning
426 genes. A total of 53 wing patterning genes were found to be expressed in the brain, while
427 50 were expressed in the eyes (S38 Table). Although none of these wing patterning genes
428 exhibited sex-specific expression (meaning only expressed in one sex) in either tissue, 46
429 were in common across the two tissues. Seven genes showed brain-specific expression,
430 including homologs for cortex (*BANY.1.2.g04256*), engrailed (*BANY.1.2.g14935*), CD63-
431 antigen (*BANY.1.2.g12556*), aristaless (*BANY.1.2.g21346* and *BANY.1.2.g24453*), and
432 BarH-1 (*BANY.1.2.g19326* and *BANY.1.2.g22154*), while four exhibited eye-specific
433 expression, including homologs for hedgehog (*BANY.1.2.g04016*) and CD63-antigen
434 (*BANY.1.2.g20540*, *BANY.1.2.g25497*, and *BANY.1.2.g25594*).

435 Several wing patterning genes were identified as differentially expressed for
436 various contrasts, including between and within sexes, in both tissue types. For the naïve
437 female vs. male brain contrast, sal-like protein 1 (*BANY.1.2.g09547*) and CD63 antigen-
438 like (*BANY.1.2.g23713*) are both upregulated in females (Fig. 5, Table S4). In the trained
439 female vs. male brain contrast protein apterous-like isoform X2 (*BANY.1.2.g08342*) is
440 upregulated in males (Fig. 5, S5 Table). In the naïve female vs. male eye contrast CD63
441 antigen-like (*BANY.1.2.g25497*) is upregulated in females (Fig. 5, S6 Table). Moreover,
442 in the eye interaction contrast CD63 antigen-like (*BANY.1.2.g25497*) is upregulated in
443 trained females and naïve males (Fig. 5, S13 Table), and in the trained vs. naïve eye
444 controlling for sex contrast CD63 antigen-like isoform X2 (*BANY.1.2.g10818*) is
445 upregulated in naïve eyes (Fig. 5, S15 Table).

446

447



448

449

450 **Figure 5: Genes that influence *B. anynana* wing patterns are also differentially expressed in**
 451 **the brain and eye during training.** Top panel contains box plots of differentially expressed
 452 genes in different contrasts. Bottom panel indicates the elements of butterfly wing pattern (gold
 453 ring, eye spot center, black ring, whole eye spot, or dorsal/ventral identity) influenced by the
 454 corresponding differentially expressed gene. For top panel, light hue = naïve, dark hue = trained,
 455 orange = male, blue = female, green = condition (general trained/naïve).

456

457

458

459

460 When comparing within sexes, three known wing patterning genes were
 461 differentially expressed in male brains or eyes. In the trained vs. naïve male brain
 462 contrast, protein bric-a-brac 2-like isoform X4 (*BANY.1.2.g17823*) is upregulated in
 463 trained males while Homeobox protein invected (*BANY.1.2.g18817*) is upregulated in
 464 naïve males (Fig. 5, S10 Table). By contrast, in the trained vs. naïve male eye comparison
 465 protein apterous-like isoform X2 (*BANY.1.2.g08342*) is upregulated in trained males (Fig.
 466 5, S11 Table). No known wing pattern genes were differentially expressed in female-
 467 specific contrasts.

468

469 **Discussion**

470 Here we identified a number of genes that were differentially expressed in the
471 brains and eyes of females and males during an imprinting-like learning event, as well as
472 several associated gene networks. We found DEGs in both tissue types, suggesting that
473 imprinting-like learning, and sexually dimorphic aspects of this learning process, are
474 associated with transcriptional change in both the peripheral sensory system and the
475 brain. A number of chemosensory genes were upregulated in females relative to males,
476 supporting the hypothesized female-specific use of pheromones in the mate preference
477 learning process (Westerman and Monteiro, 2013; Westerman et al., 2014). Furthermore,
478 a suite of butterfly wing patterning genes, which have long been hypothesized to also
479 influence mate preference and potentially serve as “magic genes,” were also differentially
480 expressed in the eyes and brains of *B. anynana* butterflies during training events, further
481 supporting their hypothesized role in mate preference and speciation.

482 One of the more interesting aspects of sexually dimorphic imprinting-like learning
483 in *B. anynana* is the presence/absence of sex pheromones in males versus females.
484 Previous studies have shown that the male sex pheromone is an indicator of age
485 (Nieberding et al., 2012), is species-specific (Bacquet et al., 2015; Nieberding et al.,
486 2008), is equally weighted with visual signals during female mate selection (Costanzo
487 and Monteiro, 2007), and influences the valence females learn to associate with visual
488 signals during imprinting-like learning (Westerman and Monteiro, 2013). Thus, male
489 chemical cues are known to be important for female mate choice in this system. On the
490 other hand, a sex pheromone has not been discovered in female *B. anynana*, and it

491 remains unclear what unconditioned stimulus males use to assign positive valence to
492 number of hindwing spots. The results of this study appear to support this sex-specific
493 use of olfactory signals during the learning process. The most clear-cut finding
494 supporting this hypothesis is that chemosensory genes are upregulated in females relative
495 to males during the training period. A second result that may be related to the differential
496 use of olfactory cues during the learning (and mate choice) process is that we found a
497 larger set of gene networks associated with the training condition in the brains and eyes
498 of males than in females. This could be a result of imprinting-like learning being more
499 consistent in males than females (Westerman et al., 2012; Westerman et al., 2014).
500 However, it could also be a side effect of females relying more heavily on olfactory
501 signals than males, as we did not include antennae in our analyses and consequently may
502 have missed learning-associated gene networks that reside in female antennae. Female
503 *Heliconius melpomene* and *Heliconius cydno* butterflies are sensitive to male pheromones
504 (Byers et al., 2020) and exhibit different antennae expression profiles before and after
505 copulation as well as sex-specific expression profiles (van Schooten et al., 2020). It
506 would be interesting to see if *B. anynana* females exhibit training-specific, sexually
507 dimorphic antennae expression profiles that correspond to their sex-specific emphasis on
508 olfactory signals during the preference learning and mate selection process.

509 While the gene expression patterns of the antennae are unknown for these
510 animals, we did find training-specific, sexually dimorphic gene expression patterns in *B.*
511 *anynana* eyes. Because female and male *B. anynana* butterflies learn preferences for
512 different visual signals and exhibit visual learning biases in different directions (gains and
513 losses, respectively (Westerman et al., 2012; Westerman et al., 2014)), one of our

514 hypotheses was that we would see sexually dimorphic expression of vision-related genes
515 during the learning process, especially in the eyes. Although we did not observe
516 differential expression of any opsins, we did find sex-dependent expression patterns of a
517 number of vision-related genes, including an ommochrome-binding protein, retinol
518 dehydrogenase 11, rhodopsin kinase 1 (*Gprk1*), and arrestin homolog isoform X2.
519 Ommochrome pigments act as filtering pigments in the eyes of butterflies, limiting the
520 wavelengths of light a butterfly can see (Arikawa and Stavenga, 2014; Stavenga, 2002).
521 These filtering pigments are sexually dimorphic in a number of different species,
522 including *H. cydno*, *H. melpomene*, *Heliconius pachinus*, and *Colias erate*, and are
523 hypothesized to influence mate choice in these systems (Buerkle et al., 2022; Ogawa et
524 al., 2013). It remains unclear whether filtering pigment type or distribution is sexually
525 dimorphic in *B. anynana*, or whether filtering pigment production or distribution in the
526 eye is plastic in response to circadian rhythms, social scenario, or age. However, our
527 findings of socially-dependent expression patterns of ommochrome-binding protein and a
528 number of other vision-related genes suggest that vision is highly dynamic, not just in the
529 context of light environment (Obara et al., 2008; Sakai et al., 2018; Wright et al., 2020)
530 and circadian rhythms (Li et al., 2008; Li et al., 2005), but also in response to social
531 environment.

532 In addition to finding vision-associated differentially expressed genes, a number
533 of learning and memory genes were differentially expressed specifically in the eyes,
534 including dopamine receptors. Moreover, the most highly connected gene for a gene
535 network associated with training condition in male eyes (the black module) encodes a
536 component of the receptor for the neurotransmitter GABA, gamma-aminobutyric acid

537 type B receptor subunit 2. This network also contained a variety of genes involved with
538 neural processing that were differentially expressed between trained and naïve male eyes,
539 including additional neurotransmitter receptors (acetylcholine receptor subunit alpha-like
540 2, gamma-aminobutyric acid type B receptor subunit 2 isoform X3, and dopamine
541 receptor 2-like). While there is some debate over whether eyes should be considered part
542 of the peripheral nervous system or the central nervous system in vertebrates (London et
543 al., 2013), there has been less attention given to the potentially broad cognitive role of the
544 retina in comparison to the optic lobe in insects (as illustrated by (Perry et al., 2017)).
545 Our findings not only indicate that transcription in the butterfly eye changes in response
546 to social scenario (presence/absence of a sexually mature conspecific), but that this
547 change includes the transcription of genes associated with higher processing, indicating
548 that neurogenomic processes associated with cognition might not be limited to the optic
549 lobe and central brain in insects, but might also occur in the retina.

550

551 *Broad role of sensory receptors and neurotransmitters in sexually dimorphic behavior*

552 Although neurogenomic assessment of sexually dimorphic behavior is relatively
553 rare to date, similarities between our results and those in other animal systems suggest
554 common mechanisms may underlie sexually dimorphic behavior across animal taxa.
555 Sensory receptors seem to be especially important and connected to downstream sexually
556 dimorphic gene networks. For example, odorant receptor expression influences female
557 receptivity and male ability to differentiate between the sexes in *D. melanogaster* (Datta
558 et al., 2008), male and female zebra finches exhibit different brain gene expression
559 profiles when listening to the same song (Gobes et al., 2009), a number of butterfly

560 species exhibit sexually dimorphic opsin expression patterns (Buerkle et al., 2022;
561 Everett et al., 2012), and *B. anynana* exhibit sexually dimorphic chemical receptor
562 expression during a mate preference learning event (this study). Sexually dimorphic
563 catecholamine-associated expression (receptors or binding proteins, for example) also
564 appears to be important for driving sexually dimorphic social behaviors across taxa, as
565 illustrated by sex-dependent distribution of tyrosine hydroxylase in male and female
566 plainfin midshipman fish brains (Goebrecht et al., 2014) and sexually dimorphic
567 association of dopamine receptors and binding proteins with social interactions in *B.*
568 *anynana* butterflies (this study). Pathways integrating sensory receptors and
569 catecholamine neurotransmitters may be particularly fruitful for future study of sexually
570 dimorphic behaviors across animal taxa.

571

572 *Wing patterning genes may be “magic” genes*

573 While butterfly wing patterning genes have long been hypothesized to play a role
574 in shaping both wing pattern and preference for wing pattern (Kronforst and Papa, 2015;
575 Kronforst et al., 2006; Merrill et al., 2015; Merrill et al., 2019; Naisbit et al., 2001),
576 evidence supporting this hypothesis has been rare. Here we show that a number of wing
577 patterning genes are differentially expressed in the brain and eyes during a sexual
578 (training) encounter. Not only are these genes associated with wing patterning in a range
579 of butterfly species, but a subset of these genes are specifically associated with aspects of
580 eyespot production in *B. anynana* (Brunetti et al., 2001; Ozsu and Monteiro, 2017;
581 Prakash and Monteiro, 2018) and/or with UV reflectance (Ficarrotta et al., 2022).
582 Because male and female *B. anynana* learn preferences for eyespot number, and

583 specifically the UV-reflective center of the eyespots (Westerman et al., 2012; Westerman
584 et al., 2014), these genes that both influence eyespots or UV scale production and are
585 differentially expressed in the brain or eyes during an intersexual social encounter
586 (*invected*, *spalt*, *apterous*, *CD63 antigen-like*, and *bric-a-brac*) are particularly promising
587 candidate magic genes in the *B. anynana* system. The brain and eye expression profiles of
588 genes known to influence wing patterning traits important for mate selection in other
589 butterfly systems, such as *BarH-1* (Woronik et al., 2019), *artistaless* (Westerman et al.,
590 2018), *cortex* (Nadeau et al., 2016), and *doublesex* (Kunte et al., 2014), support the
591 hypothesis that these genes may be expressed in the brains or eyes of the butterfly species
592 using these genes to control wing pattern elements under sexual selection. Future studies
593 should explore the pervasiveness of genes influencing both wing pattern and mate
594 preference across the Lepidoptera.

595

596 **Conclusions**

597 Here we show that sexually dimorphic, imprinting-like learning is associated with
598 sexually dimorphic gene expression in the brains and eyes of *B. anynana* butterflies
599 during a training event. Differentially expressed genes include sensory receptors and
600 genes associated with neurotransmitters in both tissue types, indicating dynamic and sex-
601 specific responses to social scenario in both the peripheral and central nervous systems.
602 Sexually dimorphic expression of chemosensory genes supports the role of pheromones
603 in female but not male imprinting-like learning, while the learning-related expression of
604 numerous wing patterning genes highlight the potential for these genes to influence both
605 wing pattern and mate preference. Future research should explore the gene and neural

606 networks bridging sexually dimorphic sensory receptors to sexually dimorphic behavior,
607 and determine the functional role of wing patterning genes in mate preference in other
608 lepidopterans.

609

610 **Materials and Methods**

611 *Study Species and Husbandry*

612 *Bicyclus anynana* is a sub-tropical African butterfly that has been reared in the lab
613 since 1988. The colony at the University of Arkansas was established in spring 2017
614 from ~1,000 eggs derived from a population in Singapore. Butterflies at the University of
615 Arkansas were reared in a climate-controlled greenhouse at ~27°C, 70% humidity, and
616 under a 13:11h light:dark cycle to mimic wet season conditions and ensure development
617 of the wet season phenotype (Brakefield and Reistma, 1991). Butterflies bred in the
618 laboratory have levels of genetic diversity comparable to those in natural populations, as
619 suggested by similar single-nucleotide polymorphism frequencies found in laboratory and
620 natural populations (Beldade et al., 2006; de Jong et al., 2013).

621 All adult butterflies used in this study hatched from eggs laid on young corn
622 plants (*Zea mays*) in breeding colony cages containing ~200-500 male and female *B.*
623 *anynana* butterflies. Plants with eggs were moved to cages containing additional corn
624 plants for larval consumption, and larvae were fed *ad libitum* until pupation. Upon
625 pupation, pupae were placed in mesh cages (31.8 cm × 31.8 cm × 31.8 cm; Bioquip,
626 Compton, CA, USA) until emergence. Upon emergence, butterflies were transferred to
627 sex- and age-specific cages to isolate the sexes from one another. All butterflies were
628 provided with fresh banana every other day.

629

630 *Behavioral assays and sample collection*

631 All behavioral assays and sample collection took place between November 2018 -
632 July 2019. Within one hour of dawn, assays were conducted by placing butterflies in a
633 novel mesh cage (39.9 cm × 39.9 cm × 59.9 cm; Bioquip, Compton, CA, USA) for a
634 three-hour observation period (Fig. 1A). Training behavioral assays consisted of either:
635 (1) a newly emerged male paired with a two-day-old, zero-spot female, for which black
636 paint (Enamel Glossy Black 1147, Testors, Rockford, IL, USA) was applied directly on
637 top of her two dorsal hindwing eyespot pupils to block all UV reflectance (for details see
638 (Westerman et al., 2014)) or (2) a newly emerged female paired with a two-day-old, four-
639 spot male, for which UV-reflective paint (White, FishVision, Fargo, ND, USA) was
640 applied between the two natural dorsal forewing eyespot pupils to create two extra
641 eyespot pupils (for details see (Westerman et al., 2012)). The UV-reflective paint closely
642 replicated the reflectance spectra of natural *B. anynana* eyespot pupils (Westerman et al.,
643 2012). All eyespot manipulations were performed one day prior to behavioral watches.
644 Control assays consisted of either one newly emerged male or one newly emerged female
645 placed in a novel mesh cage (39.9 cm × 39.9 cm × 59.9 cm; Bioquip, Compton, CA,
646 USA) in isolation. For any given training assay, a control assay using the same sex as the
647 training assay focal animal was conducted concurrently (e.g., for a newly emerged male +
648 zero-spot female training assay, a control assay consisting of a newly emerged male in
649 isolation was run in tandem). All behaviors exhibited by the observed butterflies were
650 recorded using SpectatorGo! (BIOBSERVE; Bonn, Germany). Observed behaviors

651 included: *flutter*, *fly*, *walk*, *rest* (wings closed), *bask* (wings open greater than 45°),
652 *antenna wiggle*, *court* (as defined in (Nieberding et al., 2008), and *copulate*.

653 After the three-hour behavioral watch, each butterfly's head was removed with
654 RNase-free scissors, transferred into a RNase-free microcentrifuge tube (Biotix; San
655 Diego, CA, USA), and immediately flash frozen in liquid nitrogen. Frozen samples were
656 then stored in a -80°C freezer until dissection and RNA extraction. We collected the
657 heads of ten individuals per group (trained male, trained female, naïve male, and naïve
658 female) to account for variation in response to training, as previous studies suggest that
659 ~75% of females and ~80% of males learn to prefer the trainer phenotype after a three-
660 hour training exposure (Westerman et al., 2012; Westerman et al., 2014).

661

662 *RNA extraction and cDNA library preparation*

663 To prevent RNA degradation during processing, heads were immersed in 500 µL
664 of pre-chilled RNAlater-ICE (Ambion; Austin, TX, USA) and incubated at -20°C for
665 approximately 18 hours prior to dissection. Thawed heads were then dissected under a
666 dissecting microscope (Zeiss Stemi 508; Jena, Germany) while submerged in RNAlater-
667 ICE to isolate eye and brain tissue. The eyes and brain for each sample were
668 mechanically disrupted separately in lysis buffer using RNase-free, disposable pestles,
669 and small (<200 nucleotides) and large (>200 nucleotides) RNA were extracted
670 separately for each tissue with the NucleoSpin® miRNA kit (Macherey-Nagel; Düren,
671 Germany). RNA quality and quantity were determined using a NanoDrop 2000 (Thermo
672 Fisher Scientific; Waltham, MA, USA), Qubit 2.0 (Invitrogen; Waltham, Massachusetts,
673 USA), and TapeStation 2200 (Agilent; Santa Clara, CA, USA).

674 Libraries were prepared for the eyes (left and right eye together; n=40) and brain
675 (n=40) for each individual using the KAPA mRNA HyperPrep Kit and Unique Dual-
676 Indexed Adapters (KAPA Biosystems; Wilmington, MA, USA), with 100 ng of large
677 RNA as input. After running all cDNA libraries on a TapeStation 2200 (Agilent; Santa
678 Clara, CA, USA) and confirming that they were of high quality, libraries were shipped to
679 the University of Chicago Genomics Facility on dry ice. All libraries were subjected to an
680 additional quality assessment using a 5300 Fragment Analyzer (Agilent; Santa Clara, CA,
681 USA), followed by 50 bp SE sequencing across eight lanes of a HiSeq 4000 (Illumina;
682 San Diego, CA, USA).

683

684 *Read trimming, alignment, and quantification*

685 We concatenated the raw fastq files from all eight lanes for each library and
686 performed an initial quality assessment using FastQC v0.11.5
687 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). One sample (TME_A3, a
688 trained eye sample) failed to sequence properly, so was discarded from downstream
689 analysis. Trimmomatic v0.38 was used to remove any Illumina sequencing adapters from
690 the raw reads (Bolger et al., 2014). We then aligned the adapter-trimmed reads for each
691 sample to the most recent *B. anynana* reference genome (v1.2; (Nowell et al., 2017))
692 using STAR v2.7.1a (Dobin et al., 2013) and quantified all reads using the "--quantMode
693 GeneCounts" option, which is equivalent to counts produced by the htseq-count script
694 from HTSeq (Anders et al., 2015).

695

696 *Differential gene expression analyses*

697 The read counts generated by STAR were used as input for the DESeq2 v1.24.0
698 package (Love et al., 2014) for R (Version 3.6.2, R Foundation for Statistical Computing,
699 Vienna, Austria) to conduct differential expression analyses. Specifically, we used the
700 generalized linear model design:

$$701 \quad y \sim \text{sex} + \text{condition} + \text{sex}:\text{condition}$$

702 where expression (y) is a function of *sex* (male or female), *condition* (trained or naïve),
703 and their interaction (*sex:condition*). With this design, we made five different tissue-
704 specific comparisons: (1) naïve females vs. naïve males; (2) trained females vs. trained
705 males; (3) trained females vs. naïve females; (4) trained males vs. naïve males; and (5)
706 the interaction of sex and condition. To investigate the overall effect of training while
707 controlling for differences in expression specific to sex, we performed an additional
708 tissue-specific analysis that utilized the design:

$$709 \quad y \sim \text{sex} + \text{condition}$$

710 Only genes with ≥ 10 total mapped reads were used for the differential expression
711 analyses. Gene expression comparisons were conveyed as the binary log of the
712 expression fold change ($\log_2\text{FC}$), with $\log_2\text{FC}$ shrinkage performed using the ash method
713 (Stephens, 2017) to obtain more accurate estimates of effect size. Genes were considered
714 differentially expressed if they had a false discovery rate (FDR; (Benjamini and
715 Hochberg, 1995)) < 0.05 .

716 In addition to these standard differential expression analyses, we also performed
717 permutation tests similar to those utilized in Ghalambor et al. (2015) and Bloch et al.
718 (2018). Because this method does not assume gene independence (an unlikely assumption
719 given the nature and abundance of gene co-expression networks), the risk of over-

720 correction is reduced compared to other multiple testing correction methods, resulting in
721 a more accurate representation of the expression data structure (Slonim, 2002). For each
722 tissue, we randomly assigned both the sex and treatment for each sample to create 1,000
723 permuted sample phenotype tables. For each of the reassigned sample sets, we ran the
724 DESeq2 analysis exactly as we had for the original analysis, ultimately resulting in a null
725 distribution of 1,000 p-values for every gene. For any given gene, if the p-value from the
726 original analysis was less than the 1% tail of the permuted null distribution, it was
727 considered differentially expressed. Annotations for all differentially expressed genes,
728 including the identified putative vision- and chemosensory-related gene annotations, were
729 extracted from the *B. anynana* reference genome functional annotation from (Ernst and
730 Westerman, 2021).

731

732 *Weighted gene co-expression network analyses*

733 We performed separate weighted gene co-expression network analyses
734 (WGCNA) for the brain and eyes using the WGCNA v1.70-3 R package (Langfelder and
735 Horvath, 2008) following the WGCNA package developers' recommendations. We first
736 preprocessed the expression data by removing all genes with <10 reads in >90% of the
737 samples to minimize noise from lowly-expressed genes, and a variance-stabilizing
738 transformation was performed on the remaining data using the
739 “varianceStabilizingTransformation” function in DESeq2. Signed co-expression
740 networks for each tissue were constructed by building an adjacency matrix with type =
741 “signed,” topological overlap matrix (TOM) with TOMType = “signed,” and the soft-
742 thresholding power set to 12 for brains and 14 for eyes. We then identified modules of

743 co-expressed genes using the “cutreeDynamic” function with the following parameters:
744 deepSplit = 2, pamRespectsDendro = FALSE, and minClusterSize = 30. After initial
745 module identification, we merged modules of high co-expression similarity by first
746 calculating and clustering their eigengenes (the first principal component of a module
747 representing its gene expression profile (Langfelder and Horvath, 2008)) and employing
748 the “mergeCloseModules” function with the “cutHeight” set to 0.25.

749 To identify modules that were significantly associated with any of the sample
750 traits, we used the “binarizeCategoricalVariable” function to create pairwise binary
751 indicators (“traits”) for our contrasts of interest (i.e., naïve male vs. naïve female, trained
752 female vs. naïve female, trained male vs. naïve male, and trained female vs. naïve
753 female) and correlated eigengenes with these sample traits. We then adjusted all p-values
754 using the FDR method (Benjamini and Hochberg, 1995), and any module-trait
755 correlations with an FDR <0.05 were considered significant. For all modules that showed
756 significant associations with sample traits, hub genes (genes with the highest
757 connectivity) were identified using the “chooseTopHubInEachModule” function.

758 For visualization and further analysis, both networks were then exported to
759 Cytoscape v3.8.2 (Shannon et al., 2003) using the “exportNetworkToCytoscape” function
760 with “threshold” set to 0.02. The Cytoscape “Network Analyzer” tool was used to obtain
761 further statistics regarding the connectivity of genes within the network. Specifically, we
762 calculated three statistics for each gene: (1) degree (the number of other genes connected
763 to a given gene, with a larger number indicating a more highly connected gene), (2)
764 neighborhood connectivity (the average connectivity of all of a gene’s neighboring
765 genes), and (3) clustering coefficient (how connected a gene is to its neighboring genes

766 relative to how connected it could be, with “0” representing completely unconnected and
767 “1” representing maximum connectivity).

768

769 *Gene Ontology Analyses*

770 To facilitate the characterization of DEG sets and significant modules, GO
771 enrichment analyses were performed using the Fisher’s Exact Test function in Blast2GO
772 v5.2.5 (Conesa et al., 2005) with the GO annotations extracted from Ernst and
773 Westerman (2021). In each case, all genes in the expression set (for the WGCNA
774 analysis, all genes that were used in the co-expression analysis) for the respective tissue
775 were used as the reference set, and an FDR threshold of <0.05 was set to identify
776 significantly enriched GO terms. All DEG sets and significant modules were tested for
777 GO enrichment.

778 To further explore the differences between male and female tissues for each
779 condition, we used GOExpress v1.20.0 (Rue-Albrecht et al., 2016) to identify GO terms
780 that best classify the samples from two groups (e.g., female trained brains and male
781 trained brains) based on their gene expression profiles. For these analyses, reads were
782 first normalized to counts per million (CPM) with edgeR v3.28.1 (Robinson et al., 2010),
783 and only genes with ≥ 1 CPM for at least 10 samples (the maximum number of replicates
784 per group) were retained for the input expression matrix. The random forest was set to
785 10,000 trees, and GO terms that were associated with at least five genes and with a p-
786 value <0.05 after 1,000 permutations were considered significant.

787

788 *Identification of wing patterning genes*

789 In addition to examining differential expression, co-expression networks, and GO
790 signatures, we also investigated the expression patterns of known wing patterning genes,
791 as these genes have been hypothesized to act as “magic genes” and to have the capacity
792 to influence both preference as well as the preferred trait (Servedio, 2009; Smadja and
793 Butlin, 2011; Westerman, 2019). Specifically, we used the functional annotations and
794 butterfly wing patterning gene list from Ernst and Westerman (2021) to identify wing
795 patterning genes expressed in eye and brain tissue and to determine if they were
796 differentially expressed between the sexes. The genes included numerous *B. anynana*
797 wing patterning genes (Beldade and Peralta, 2017; Bhardwaj et al., 2018; Connahs et al.,
798 2019; Matsuoka and Monteiro, 2018; Monteiro et al., 2013; Monteiro et al., 2006;
799 Monteiro and Prudic, 2010; Ozsu et al., 2017; Prakash and Monteiro, 2018, 2020; Saenko
800 et al., 2011), as well as genes characterized in other butterfly species (Ficarrotta et al.,
801 2022; Martin and Reed, 2010; Nadeau et al., 2016; Reed et al., 2011; Westerman et al.,
802 2018; Woronik et al., 2019).

803

804 *Analysis of Behavior*

805 We first conducted a Shapiro-Wilk test to assess normality of the behavioral data.
806 We then performed a Kruskal-Wallis test to examine the effect of sex on behavior,
807 followed by a second Kruskal-Wallis test subset by treatment (naïve, trained, trainer) to
808 test for the effect of sex on behavior in each treatment. We conducted a principal
809 components analysis (PCA) on behavior to search for hidden correlations and create new
810 composite variables (S39 Table). We then performed a Kruskal-Wallis test to test for the

811 effect of sex on PC1, PC2, and PC3. We calculated a Bonferroni correction to account for
812 multiple testing, producing an adjusted significance value of $p = 0.0025$.

813

814 *Ethical Note*

815 All *B. anynana* butterflies were maintained in laboratory conditions as specified
816 by U.S. Department of Agriculture APHIS permit P526P-17-00343. Butterflies not used
817 for this experiment were maintained with ample food and water until natural death.

818

819 **Acknowledgements**

820 We thank Matthew Murphy, Grace Hirzel, Deonna Robertson, and Sushant Potdar
821 for assistance with animal husbandry. This research was supported by NSF IOS grant
822 #1937201 to ELW, an Arkansas Biosciences Institute (the major research component of
823 the Arkansas Tobacco Settlement Proceeds Act of 2000) grant to ELW, a University of
824 Arkansas Honors College grant to GAA & ELW, the Arkansas High Performance
825 Computing Center, which is funded through multiple NSF grants and the Arkansas
826 Economic Development Commission, and the University of Arkansas.

827

828 **Competing Interests:** The authors declare no competing interests.

829

830 **Data Availability:** All raw sequence data associated with this study are accessible
831 through the NCBI Sequence Read Archive (SRA) database under XXX. Behavioral data
832 are available at Dryad Database XXXX. All other data presented in this study are
833 available within this manuscript and its Supplemental Information.

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