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Visual mate preference evolution during butterfly speciation is linked to neural processing genes

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Many animal species remain separate not because their individuals fail to produce viable hybrids but because they “choose” not to mate. However, we still know very little of the genetic mechanisms underlying changes in these mate preference behaviours. *Heliconius* butterflies display bright warning patterns, which they also use to recognize conspecifics. Here, we couple QTL for divergence in visual preference behaviours with population genomic and gene expression analyses of neural tissue (central brain, optic lobes and ommatidia) across development in two sympatric *Heliconius* species. Within a region containing 200 genes, we identify five genes that are strongly associated with divergent visual preferences. Three of these have previously been implicated in key components of neural signalling (specifically an *ionotropic glutamate receptor* and two *regucalcins*), and overall our candidates suggest shifts in behaviour involve changes in visual integration or processing. This would allow preference evolution without altering perception of the wider environment.

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The evolution and maintenance of new animal species often relies on the emergence of divergent mating preferences^{1,2}. Changes in sensory perception or other neural systems must underlie differences in innate behaviours between species, and will ultimately have a genetic basis. Although the significance of behavioural barriers for speciation has been recognized since the Modern Synthesis³, we know little of the genes underlying changes in mating preferences, or variation in behaviours across natural populations more broadly⁴. Identifying these genes will provide an important route towards understanding how behavioural differences are generated, both during development and across evolutionary time.

Previous studies of isolating preference behaviours have largely been limited to the identification of causal genomic regions, which almost invariably contain many genes^{5–7}. Only a handful of studies have identified likely candidate genes that contribute to species behavioural preferences. These are largely limited to chemosensory-guided mating preferences^{8–10}, but see¹¹, and have identified changes at chemoreceptor genes. To our knowledge, only two studies—in incipient fish species—have identified candidates for visual preference evolution, albeit indirectly, both suggesting a role for sensory perception mediated by changes in the peripheral visual system (e.g., opsins)^{12,13}. Whether or not visual preference evolution generally involves shifts at the sensory periphery, or in downstream processing, remains unknown.

The closely related species *Heliconius melpomene* and *Heliconius cydno* differ in warning patterns, which are both under disruptive selection due to mimicry¹⁴ and are important mating cues¹⁵. As a result, these divergent patterns couple ecological and behavioural components of reproductive isolation, which (as predicted by so-called “magic trait” models¹⁶) is expected to facilitate speciation in the face of gene flow. In central Panama, *H. melpomene* shares the black, red and yellow pattern of its local *Heliconius erato* co-mimic, whereas *H. cydno* shares the black and white patterns of *Heliconius sapho*. *H. melpomene* and *H. cydno* remain separate largely due to strong assortative mating¹⁷. Visual preferences for divergent patterns are particularly apparent in males, which strongly prefer to court conspecific females^{15,18,19}. Differences in warning pattern between *H. melpomene* and *H. cydno* are largely due to expression differences in just three genes, specifically *optix*²⁰, *WntA*²¹ and *cortex*²².

Quantitative trait locus (QTL) mapping of *H. melpomene* and *H. cydno* has revealed three genomic regions of major effect that influence the relative time males spend courting red *H. melpomene* or white *H. cydno* females¹⁹. Notably, the best supported QTL was in the same genomic region as *optix*, the gene responsible for presence and absence of red colour pattern elements in *Heliconius*²⁰. Genetic linkage is expected to facilitate speciation by impeding the breakdown of genetic associations between ecological and mating traits^{22,23}. Nevertheless, this QTL, and its associated candidate region, contain hundreds of genes, and the exact genes responsible for differences in preference behaviour are not known.

Here, we first confirm that the behavioural QTLs identified previously are associated with variation in male courtship initiation. We then identify genes within the major QTL, which were differentially expressed in the neural tissue (central brain, optic lobes and ommatidia) of *H. melpomene* and *H. cydno*, or have protein-coding changes predicted to alter protein function. Out of 200 genes within the QTL region, we identify just five candidates likely to underlie assortative mating behaviours.

Results

Chromosome 18 is associated with courtship initiation. Our previous results reveal that QTLs on chromosomes 1, 17 and 18

influence the relative time hybrid males spend courting red *H. melpomene* or white *H. cydno* females¹⁹. However, the time males spend courting a particular female might depend not only on male attraction, but on the female’s response (and in turn his response to her behaviour). To confirm that these previously reported QTLs influence male approach behaviours (as opposed to other traits that may influence courtship, for example male morphology²⁴), we reanalyzed our previous data, explicitly considering whether males initiated courtship towards *H. melpomene*, *H. cydno* or both types of female during choice trials.

Consistent with our previous analyses¹⁹, we found that males of both species, and in particular *H. melpomene* males, show a strong preference for females of their own phenotype. F1 and backcross-to-*melpomene* prefer to court *melpomene* females, whereas courtship initiation behaviours segregate in the backcrosses to *cydno* (Fig. 1). The QTL on chromosome 1 was retained in our model of initiation behaviours (Supplementary Fig. 1; $n = 139$, $\Delta\text{ELPD} = -13.6$ (SE ± 5.7), i.e., a change of 2.34 SE units). In contrast, the QTL on chromosome 17 was not retained ($n = 139$, $\Delta\text{ELPD} = -2.1$ (SE ± 3.0)). Notably, backcrosses-to-*cydno* males heterozygous (i.e., with a *H. melpomene* allele derived from the F1 father) at the best supported QTL from our previous analysis¹⁹ (on chromosome 18) initiated courtship towards *H. melpomene* females more frequently than males homozygous for the *H. cydno* allele (Fig. 1, bottom left; $n = 139$, $\Delta\text{ELPD} = -10.9$ (S.E. ± 5.1), i.e., a change of 2.14 SE units). As in our previous analyses of relative courtship time¹⁹, we found little evidence for an interaction between the QTLs on chromosome 1 and 18 (including the interaction led to a worse fit compared to a model excluding the interaction: $\Delta\text{ELPD} = -1.7$ (SE ± 0.9), i.e., a change of 1.89 SE units), suggesting additive effects. Together with previous evidence that male hybrids bearing *H. melpomene* alleles at *optix* prefer to court the artificial models of *H. melpomene* females over those of *H. cydno*²⁵, these results suggest that the QTL on chromosome 18 harbours genes for visual attraction behaviours towards females with the red pattern (and that the *H. melpomene* alleles are dominant). As a result, we focused our subsequent analyses on this QTL on chromosome 18 (and also because tight linkage of *optix* allowed us to track the alleles at the preference locus in hybrid crosses). A recent study²⁶ also reports no candidate chemosensory genes for reproductive isolation at this QTL region on chromosome 18 (or at any other QTL).

27 genes within the major QTL are differentially expressed. We hypothesized that changes in gene regulation that determine differences in visual mate preference behaviours might occur during pupal development (for instance, during visual circuit assembly) or at the adult stage, and must involve changes in the peripheral and/or central nervous system²⁷. Therefore, we generated RNA-seq libraries for combined eye and brain tissue, across two pupal stages (around the time of ommochrome pigment deposit and halfway through pupal development) and one adult stage, for *H. melpomene* and *H. cydno* and compared their gene-expression levels. Across the QTL region on chromosome 18 (which spans 2.75 Mb and contains 200 genes), we identified 27 genes that show differential expression between *H. melpomene* and *H. cydno*, in at least one of the three developmental stages. These were mostly located within the QTL peak (i.e., the genomic region with strongest statistical association with male preference) or in close proximity to *optix* (Fig. 2). The same genes were frequently differentially expressed across development (Supplementary Table 1), with 11 genes being differentially expressed in more than one stage.

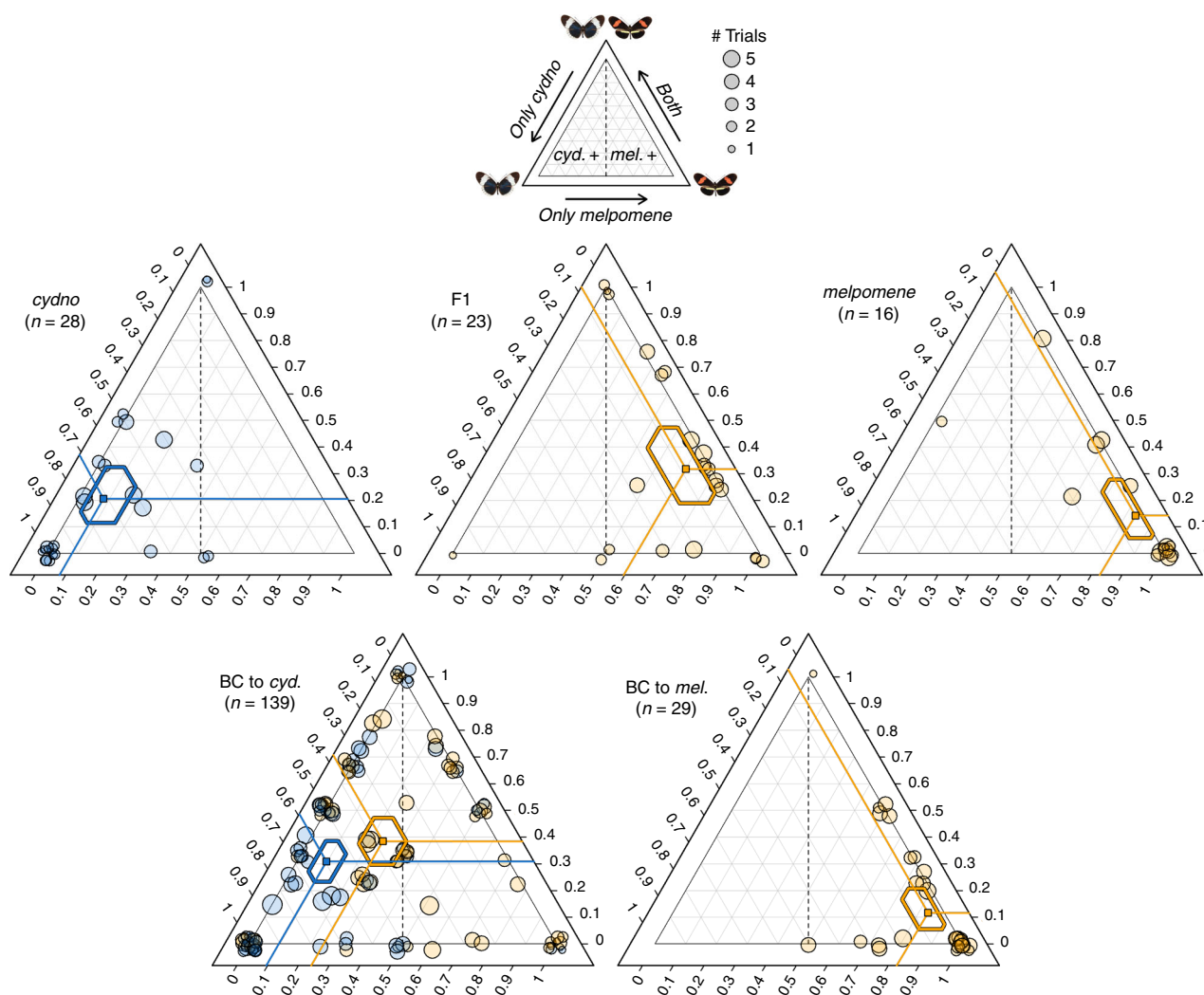


Fig. 1 Genotype at the QTL on chromosome 18 influences courtship initiation. Ternary plots showing the proportion of 15-min choice trials in which single male individuals initiated courtship towards *H. melpomene*, *H. cydno* or both females. Left ternary axis shows the proportion of trials where courtship was initiated towards *H. cydno* female only, bottom axis towards *H. melpomene* female only, and right axis towards both female species. Trials without male response were removed from the dataset. Each point represents a single individual and the location of the point in the triangle is a way of representing these three proportions at once. Lines project the three predicted proportions to corresponding values on the three axes and 95% credibility intervals (CRIs) for these proportions are shown as hexagons. Orange points represent individuals that have inherited at least one *H. melpomene* derived allele at the preference QTL on chromosome 18 (i.e., either *melp/melp* or *cyd/melp*); and blue points represent individuals that are homozygous for *H. cydno* alleles at the preference QTL on chromosome 18 (i.e. *cyd/cyd*). Point size is scaled to the number of trials in which the male showed a response and a 'jitter' function has been applied (leading to some dots being jittered to outside the triangle).

The genomic region between the start of chromosome 18 and *optix* (comprising the QTL peak) is highly divergent between *H. melpomene* and *H. cydno*²⁸, and divergent gene sequences within this region could also introduce mapping biases of RNA-seq reads. To account for this, we repeated the analysis having mapped to both the *H. melpomene* reference genome²⁹ and to a *H. cydno* genome³⁰. Generally, we found similar patterns of differential expression when mapping to the *H. cydno* genome (Supplementary Fig. 2, Supplementary Table 2A). Nevertheless, in subsequent analyses we excluded two genes, HMEL034187g1 and HMEL034229g1, which showed reversal of the fold change or did not show differential expression when mapping to the *H. cydno* genome, respectively.

Although our focus was primarily on chromosome 18, two genes within the QTL region on chromosome 1 were also differentially expressed for at least one developmental stage (Supplementary Table 1), when mapping to the *H. melpomene* and *H. cydno* genomes. Of these, one (HMEL003796g1),

associated with the regulation of enolase, was also upregulated in F1 hybrids (see below).

Regucalcin2 and Grik2 are upregulated in hybrid males. Our previous behavioural experiments suggest that the alleles for the *H. melpomene* behaviour are dominant over the *H. cydno* alleles^{19,25} (Fig. 1). Given this pattern of dominance, we predicted that genes underlying variation in male preference to be up- or downregulated in the brains of both *H. melpomene* and first generation (F1) hybrid males, with respect to *H. cydno*. Of the putative genes differentially expressed between *H. cydno* and *H. melpomene* reported above, only four, within the QTL candidate region, were differentially expressed between the F1 hybrids and *H. cydno* (Fig. 2). These included two *regucalcins* (also called *senescence marker proteins-30*: HMEL013552g1, HMEL034199g1), an *ionotropic glutamate receptor* (HMEL009992g4), which is a putative ortholog of *Grik2*, and one gene with no annotated

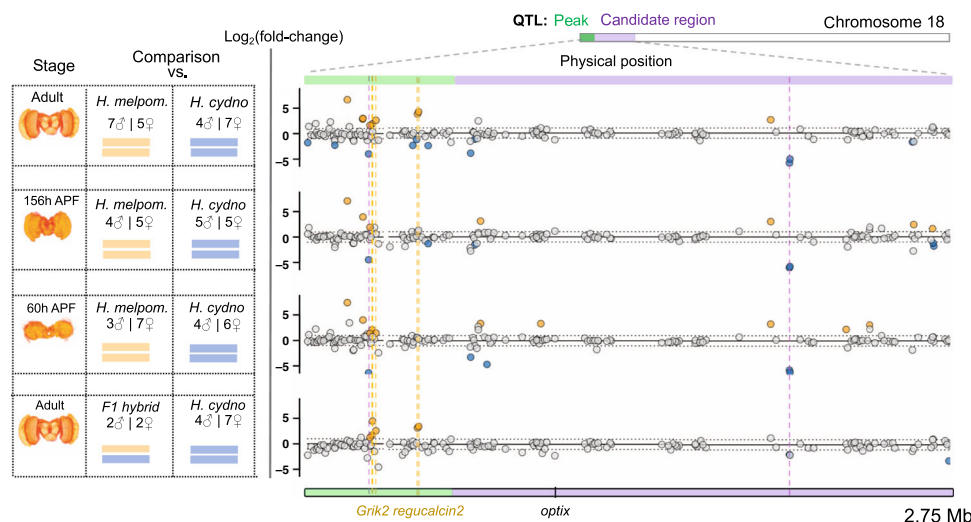


Fig. 2 Differential expression at the preference QTL region on chromosome 18. Left: Summary of the comparative transcriptomic analyses with stage, number of samples and chromosome 18 composition. Right: the corresponding results, zooming in on the QTL region on chromosome 18. The x-axis represents physical position. The QTL peak, and the rest of the QTL 1.5 LOD candidate region (from ref. ¹⁹) are shown in green and purple, respectively. Points correspond to individual genes, with the y-axis indicating the $\log_2$ (fold change) for each comparison. The two horizontal dashed lines (at y-values of 1 and -1) indicate a twofold change in expression. Genes showing a significant twofold+ change in expression level between groups are highlighted in orange and blue, where orange indicates higher levels in *H. melpomene* or in the hybrids *cyd/melp* (blue if in *H. cydno*—hybrids *cyd/cyd*). Vertical dashed lines highlight those genes that are differentially expressed between *H. melpomene* and *H. cydno* AND between *cyd/melp* vs *cyd/cyd* individuals, at the same stage (*Grik2* and *regucalcin2* at the adult stage, *Grik2* at 60 h APF). Two genes highlighted by dashed fuchsia vertical lines were excluded because they did not show differential expression, or showed reversal of the fold change when mapping RNA-seq reads to the *H. cydno* genome. Note that *Heliconius* brain (reconstruction) images, added for reference, do not include the eyes (ommatidia and retinal membrane).

function (HMEL009992g1). We obtained the same results regardless of whether we considered both males and females together, or males alone. Further inspection of spliced mRNA-reads indicated that the two annotated *regucalcins* were in fact a single gene (from now on referred to as *regucalcin2*). This was also the case for the *ionotropic glutamate receptor* and the gene with no annotated function (from now on referred to as *Grik2*).

To ensure that the apparent fragmentation of a few gene models in the *H. melpomene* (Hmel2.5) annotation³¹ did not introduce inaccuracies in estimates of differential gene expression, we produced a new transcript-based annotation of the *melpomene* genome using Cufflinks's RABT³². Repeating all comparative transcriptomic analyses using this new annotation (where exons previously considered to be distinct genes are now assigned correctly to single genes), we confirmed that both *regucalcin2* and *Grik2* were differentially expressed in both species and hybrids comparisons. Finally, to determine whether differential expression of these genes was repeatable across sequencing experiments, we generated RNA-seq libraries for additional *H. melpomene*, *H. cydno* and F1 hybrids adult brains, sampled 5 years after our initial tissue collection and sequenced independently. Differential expression of *Grik2* and *regucalcin2* is consistently detected both between *H. melpomene* and *H. cydno* and between F1 hybrids and *H. cydno*, both when these datasets are analysed separately, and when combined.

Regucalcin2 and Grik2 show cis-regulatory effects. Causal changes in gene regulation underlying phenotypic variation associated with the QTL must result from *cis*- rather than *trans*-regulation. In other words, if changes in expression of *Grik2* and *regucalcin2* account for the observed shifts in behaviour associated with the QTL these must be due to changes within the *cis*-regulatory regions of the genes themselves (as opposed to of other *trans*-acting genes elsewhere in the genome); and these causal mutations should be within the QTL region on chromosome 18.

To determine whether differences in gene-expression levels between parental species were due to *cis*- or *trans*-regulatory changes, we conducted allele-specific expression (ASE) analyses in adult F1 hybrids (from both sequencing batches). In F1 hybrids, both parental alleles are exposed to the same *trans*-environment, and consequently *trans*-acting factors will act on alleles derived from each species equally (unless there is a change in the *cis*-regulatory regions of the respective alleles). Therefore, differences in ASE indicate changes in *cis*-regulatory regions³³. For both candidate genes (*Grik2* and *regucalcin2*) the *H. melpomene* allele was significantly more highly expressed relative to the *H. cydno* allele ($p < 0.001$, Wald test), suggesting *cis*-regulatory effects (Fig. 3).

Gene-expression differences in backcross hybrids. In order to study the specific effects that *H. melpomene* derived alleles at the QTL on chromosome 18 had on gene-expression, we introgressed this region into a *H. cydno* background through multiple backcrosses (crossing design in Supplementary Fig. 3). We wanted to investigate whether differences at this QTL regulated expression of any specific genetic pathway during development, and more generally what changes in genome-wide transcription were observed in hybrids differing (mostly) just at this QTL region. We compared six *cyd/melp* vs. ten *cyd/cyd* (at the QTL region on chromosome 18) BC3 hybrids sampled at 156 h after pupal formation (APF) (Supplementary Fig. 5A), and eight *cyd/melp* vs. nine *cyd/cyd* for those at 60 h APF.

Across the entire genome, only 23 genes at 156 h APF and 29 genes at 60 h APF were differentially expressed between third-generation backcross hybrids with *cyd/cyd* and *cyd/melp* genotypes at the QTL. Within the QTL candidate region, *Grik2* was the only gene detected as differentially expressed between species and hybrids at these pupal stages (at 60 h APF, Supplementary Fig. 4). Of the remaining ten genes that were differentially expressed between both species and backcross hybrids at either

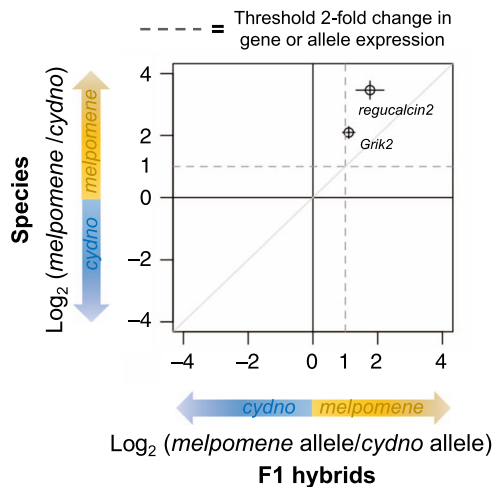


Fig. 3 *Grik2* and *regucalcin2* show evidence of allele-specific expression. Points indicate the mean value, and bars the standard error, of the (base 2) logarithmic fold change in expression between parental species (vertical) ($n = 23$ biologically independent samples) and the alleles in F1 hybrids (horizontal) ($n = 10$ biologically independent samples), for candidate genes (as defined in the transcript-guided annotation). Dashed lines indicate the threshold for a twofold change in expression for the genes in the species (horizontal), and for the alleles in the hybrids (vertical). Both genes seem to be regulated by a combination of *cis*- and *trans*-acting factors, rather than *cis*-acting factors alone (which would be indicated by $y = x$).

stage, seven were located within the introgressed region (0–6.3 Mb, Supplementary Table S3), and it seems likely that these are regulated by introgressed *H. melpomene* *cis*-acting elements. We discuss the possibility of a *cis*-regulatory element within the QTL candidate region acting on a gene outside the QTL in the Supplementary information.

It is possible that causative loci (e.g. expressed RNA or protein factors) within the QTL could act on other genes in *trans* (both on chromosome 18 and elsewhere in the genome), and identifying these could provide insight into the mode of action of causative genetic elements. Three genes differentially expressed both in species and backcross hybrid comparisons, and located outside of introgressed regions, including HMEL014795g1 and HMEL015842g1 (at 10.2 Mb and 13.5 Mb on chromosome 18, respectively) and HMEL030024g1 (on chromosome 1) might be considered good candidates for *trans*-regulation. However, in our backcrosses, the region on chromosome 18 introgressed from *H. melpomene* into a *H. cydnno* background extends ~3.6 Mb beyond the QTL candidate region, making it difficult to determine whether these genes are regulated by loci associated with variation in behaviour. Furthermore, no genetic pathway was enriched for gene-expression differences between these hybrids at either pupal stage (PANTHER enrichment test³⁴), suggesting that overall this QTL harbours a few, modular changes in gene regulation in the developing brain/eyes of *H. cydnno* and *H. melpomene*, or at least not impacting on other genes in *trans* with a clear mode of action.

To verify that differential expression of candidate genes within the QTL region is driven by *H. melpomene* alleles on chromosome 18 and not by other *H. melpomene* alleles at *trans*-acting genes on other chromosomes, we compared gene-expression levels between hybrids carrying *cyd/melp* vs. *cyd/cyd* regions on chromosomes chr1, chr4 and chr15, chr20 (Supplementary Fig. 5A). In these comparisons, there was no signal of differential expression on chromosome 18. This supports the *cis*-regulatory activity of the *melpomene* allele of candidate genes on chromosome 18. To test this further, we conducted another ASE study in

the BC3 hybrids, which suggested *trans*-only regulatory effects for *Grik2* at these pupal stages, and *cis*-regulatory effects for *regucalcin2* at 60 h APF ($p < 0.038$, Wald test) (Supplementary Fig. 6). Since causal gene/s might exert an effect on behaviour due to their action during development or in adult form, and this action might in turn be differently (*cis*- vs *trans*-) regulated, we still considered both genes as strong candidates.

Four candidate genes have protein-coding substitutions. Because shifts in behavioural phenotypes could be due to changes in protein-coding regions, we additionally considered protein-coding substitutions between *H. melpomene* and *H. cydnno*. Overall, we found 152 protein-coding substitutions, spanning 54 of the 200 genes across the entire QTL candidate region. We then studied whether these variants were predicted to have non-neutral effects on protein function with PROVEAN³⁵. The PROVEAN algorithm predicts the functional effect of protein sequence variations based on how they affect alignments to different homologous protein sequences. We found four genes with such predicted effects (PROVEAN score < -2.5): Specifically, a WD40-repeat domain containing protein (HMEL013551g3), a cysteine protease (HMEL009684g2), a MORN motif containing protein (HMEL006660g1), and another *regucalcin* (HMEL013551g4) adjacent to, but distinct from, that found to be differentially expressed above (from now on referred to as *regucalcin1*).

Candidate genes occur in regions with reduced gene flow. Of our six candidate genes for preference behaviours that contribute to reproductive isolation between *H. cydnno* and *H. melpomene* (*regucalcin2*, *Grik2* and the four genes with protein-coding modifications), five are found within the QTL peak (Fig. 4). Genetic changes causing reproductive isolation between populations are expected to reduce localized gene flow in their genomes. Therefore, we compared the position of our candidate genes to estimated levels of admixture proportions (f_d)³⁶ between *H. melpomene* and *H. cydnno* across the QTL candidate region³⁷. We found that candidate genes were located in genomic regions with low f_d values (Fig. 4), suggesting localized resistance to gene flow between *H. melpomene* and *H. cydnno* at these genes and their putative *cis*-regulatory regions.

Discussion

Behavioural isolation is frequently implicated in the formation of new species^{1,38}, and often involves the correlated evolution of both mating cues and mating preferences. Here we have analysed a genomic region in a pair of closely related sympatric butterflies, *H. cydnno* and *H. melpomene*, that contains genes for divergence in both an ecologically relevant mating cue and the corresponding preference. Physical linkage between ecological and mating traits is expected to facilitate speciation by allowing different barriers to act in concert to restrict gene flow^{39,40}. Although the genes underlying changes in the warning pattern cue in *Heliconius* are well characterized^{20–22,41} (e.g., *optix*), those underlying the corresponding shift in behaviour have not previously been identified^{19,42,43}. We have pinpointed a small number of genes that fall within the peak of the best supported QTL (on chromosome 18), which show either expression (*regucalcin2* and *Grik2*) or protein-coding differences (HMEL013551g3, HMEL009684g2, and *regucalcin1*) and fall within a region of reduced admixture, that are strong candidates for modulating mating behaviour.

Two broad neural mechanisms could underlie the evolution of divergent visual preferences, involving changes in either (1) detection at the sensory periphery or (2) the processing and/or integration of visual information. Although *H. melpomene* and *H. cydnno* have the same retinal mosaics/class of photoreceptors⁴⁴,

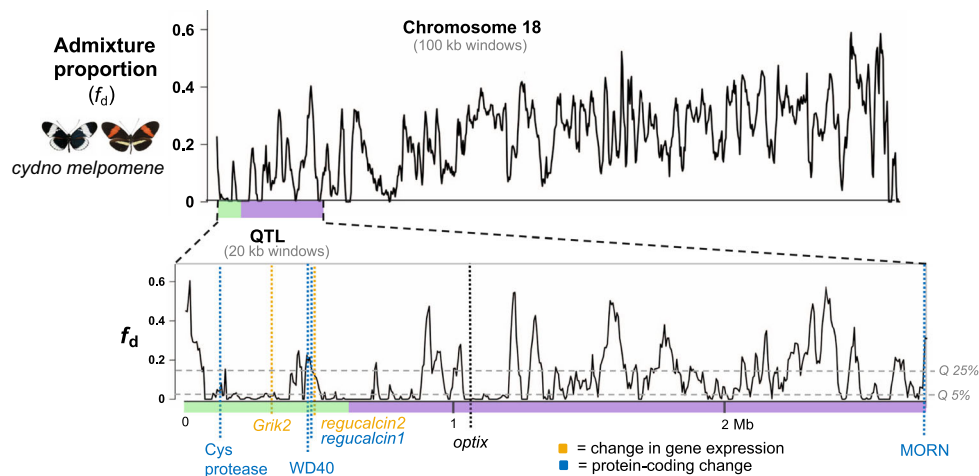


Fig. 4 Gene flow at the QTL region for behaviour. Admixture proportion (f_d) values estimated in overlapping 100 kb (top) and 20 kb (bottom) windows for chromosome 18 (top) and the QTL region (bottom) between *H. melpomene rosina* and *H. cydno chioneus*, with candidate genes positions highlighted by a vertical dashed line, and *optix* location displayed for reference. The x-axis represents physical position, the y-axis indicates the f_d value. f_d values close to zero indicate that the proportion of shared derived alleles, and consequently gene flow, between *H. melpomene* and *H. cydno* is small (or zero), implying localized selection against foreign alleles that introgress between the two species. 5 and 25% quantile of the f_d distribution are indicated by horizontal grey dotted lines.

spectral sensitivity in the *Heliconius* eyes could be altered by filtering pigments⁴⁵, or other physiological processes taking place at the photoreceptors/sensory periphery, eventually shifting sensitivity towards different wavelengths (and possibly colour patterns). It has previously been hypothesized that the gene regulatory networks for ommochrome deposition in the *Heliconius* eyes might have been co-opted in the wings⁴⁶, where *optix* plays a central role, and therefore that *optix* might play a role in eye pigmentation in *Heliconius*. However, the protein product of *optix* has not been detected in pupal or adult retinas of various *Heliconius* species tested⁴⁷, and therefore has no obvious link to ommochrome deposition in the eyes. More generally, the underlying evolutionary mechanism is unlikely to involve detection at photoreceptors, as this would probably have a broad effect on downstream processing² and alter the visual perception of the animal's wider environment.

The second mechanism, involving changes in the processing, and/or integration, of visual information, could act through an alteration of neuronal activity or connectivity. For instance, different levels of gene expression in conserved neural circuits between *H. melpomene* and *H. cydno* may affect overall synaptic weighting and determine whether a signal (e.g., colour and motion) elicits a motor pattern (response towards a female) or not. Consistent with this scenario, the composition of ionotropic receptors at post-synapses is a key modulator of synaptic transmission⁴⁸, implicating *Grik2*. Differential expression of ionotropic receptors is also associated with variation in female preference behaviours in fish^{49–51}, raising the possibility that ion channels might provide a likely route to modulate mate preferences across taxa more broadly. *Regucalcins* are involved in calcium signalling⁵², which regulates synaptic excitability and plasticity⁵³, and has an important role in axon guidance⁵⁴ (albeit alongside additional roles across a broad range of biological processes), making the two *regucalcins* we identify strong candidates for behaviour.

Changes in the regulation of genes with pleiotropic effects are likely to be less detrimental compared to changes in their protein-coding sequences⁵⁵ (although emerging evidence has begun to suggest that enhancer/repressor elements may be more pleiotropic than previously thought^{56,57}). Furthermore, there is

considerable evolutionary potential in the co-option of transcription factors/networks⁵⁵ that regulate neural patterning or neuron-type activity, possibly resulting in novel adaptive expression patterns. In line with this, *regucalcin2* and *Grik2*, which are differentially expressed in the eyes and brain in both our species and hybrid comparisons, are likely to be involved in multi-functional processes, such as calcium signalling and ion transport, and likely have pleiotropic alleles. We also found evidence of *cis*-regulatory effects for both genes, which would be required of the causal genetic change within the QTL, if it were to affect gene regulation.

Neither *regucalcin2* nor *Grik2* shows male-biased gene expression, which might be expected of candidate genes for a behaviour that is evident only in males. It is possible that the lack of sex-biased expression indicates that visual cues are similarly important in female mating preference in this species pair. Indeed, Chouteau et al.⁵⁸ report that female preferences contribute to *disassortative* mating between colour pattern morphs of *Heliconius numata*, and it is possible that in *H. cydno* and *H. melpomene* females share the same genetic basis for colour pattern-based discrimination as males. Alternatively, if visual preference behaviour is restricted to males, changes in gene expression may be integrated differently in the female and male nervous systems. The role of female preference in *Heliconius* mate choice remains poorly understood. Although emerging data suggests that female choice does contribute to reproductive isolation between *melpomene-cydno* clade taxa^{59,60}, it remains to be tested if there is a strong visual component to this preference similar to that observed in males.

Despite expectations that non-coding, regulatory loci may provide a flexible route to divergent mating preferences, we also found substitutions in coding regions at the QTL, which are predicted to have an effect on protein functioning and therefore remain strong candidates. These genes include *regucalcin1*, which is distinct from, but located next to, *regucalcin2* (which is differentially expressed). Notably, the eye transcript of *regucalcin1* was recently characterized as fast-evolving across *Heliconius* species⁶¹. Other candidates include a *cysteine protease*, which functions in protein degradation, and might be linked to behaviour for example through degradation of neurotransmitters, a

MORN motif containing protein (function unknown), and a WD40 containing protein. WD-repeat containing proteins have been implicated in a wide array of functions ranging from signal transduction to apoptosis (<https://www.ebi.ac.uk/interpro>).

Although preference for red colouration and the *optix* gene are tightly linked, we find no evidence that *optix* is differentially expressed in the eyes or brains of our two species. It is also not located within the QTL peak (and it contains no non-synonymous changes in protein-coding regions²⁰). It seems unlikely therefore that changes in cue and preference are pleiotropic effects of the same allele. More generally, although we have pinpointed the strongest candidates yet identified for assortative mating behaviours in *Heliconius*, it is possible that actual causal changes in gene regulation are restricted to developmental stages other those sampled, or restricted to a few neuronal populations not detected with transcriptomic data from eyes and whole brain tissue. Nonetheless, by sampling at two pupal stages (around the time of *optix* expression/ommochrome pigment deposit in the wing/eye and halfway through pupal development) and at the adult stage, we should have captured important transitions for the behavioural programming of the two species.

Work in the past decade has shown that complex innate behavioural differences between species can be encoded in relatively few genetic modules^{62,63}, but very few studies^{64–67} have identified specific genes underlying behavioural evolution. Traditional laboratory organisms continue to provide important insights into the evolution and genetics of behaviour^{27,65,67}; however, comparative approaches are required to determine if developmental principles can be broadly applied, and also to incorporate a wider range of phenotypic variation and sensory modalities. The challenge now is to increase the resolution of studies in non-traditional systems, in order to link individual genetic elements to behaviours, and the sensory and/or neurological structures through which they are mediated. In this light, and focussing on a genomic region that also contains a major gene for variation in warning pattern, we have identified a small handful of strong candidate genes associated with the evolution of visual mate preference behaviours in *Heliconius*. Because these genes are in tight physical linkage with the locus for the corresponding shifts in an ecologically relevant mating cue, they provide an important opportunity to investigate the build-up of genetic barriers crucial to speciation. A second QTL (on chromosome 1), not tightly linked to any known wing patterning genes, additionally influences courtship initiation in these species; although beyond the scope of the current study, it would nevertheless be interesting to investigate this region more thoroughly in future work. The candidate genes identified here seem more likely to alter visual processing or integration, rather than detection at photoreceptors, consistent with permitting changes in mate preference without altering perception of the animal's wider environment.

Methods

Courtship initiation analyses. Butterfly rearing, crossing design and genotyping are described in detail elsewhere¹⁹. In brief, we assayed male preference behaviours for *H. melpomene*, *H. cydno*, their first generation (F1) hybrids and backcross hybrids to both parental species in standardized choice trials. Males were introduced into outdoor experimental cages (1 × 1 × 2 m) with a virgin female of each species and courtship behaviours recorded. Whenever possible, trials were repeated for each male (median = 5 trials). To determine whether previously identified QTLs for courtship time contribute to variation in courtship initiation behaviours, we performed a *post-hoc* analysis using categorical models in a Bayesian framework with a multinomial error structure, using the R package *brms*. All models were run under default priors (non- or very weakly informative). In contrast to our previous analysis¹⁹, in which we considered the number of minutes (i.e., time) for which courtship was directed towards *H. cydno* or *H. melpomene* females, here the response variable was the number of trials in which male courtship was initiated towards *H. cydno* females only, *H. melpomene* females only, or both female types

(hereafter referred to as “initiation”). Across males the median number of trials with a response was 3. Using backcross-to-*cydno* males only, we fitted initiation as a response variable to genotype (*cyd/cyd* or *cyd/melp*) at each QTL, which were included as separate fixed effects. Individual ID was fitted as random factor. To test the effect of each QTL on male initiation, we compared the saturated model incorporating all three QTL with reduced models excluding each QTL in turn, using approximate leave-one-out cross-validation⁶⁸ as implemented in *brms*, and based on expected log pointwise predictive density (ELPD). Normal distribution of ELPD can be a straightforward approximation given our large samples sizes ($n = 139$)⁶⁸. Therefore, we considered an absolute value of ELPD greater than 1.96 units of its standard error as indicative of the reduced model being less-informative than the saturated model (95% confidence). Males that did not initiate courtship to any female across trials were excluded from the analyses, resulting in a dataset of 139 males, from a total of 146 backcross males for which we had genotype data. Finally, we extracted predictors and credibility intervals for backcross males with differing genotypes from the minimum adequate model. Credibility intervals for *H. melpomene*, *H. cydno*, F1 hybrid and backcross to *melpomene* males displayed in Fig. 1 were generated following the same procedures. Raw data and analysis code are available in the following github repository: https://github.com/SpeciationBehaviour/neural_genes_heliconius.git.

Butterfly collection, rearing and crossing design for expression analyses. Wild *H. melpomene rosina* and *H. cydno chioneus* individuals were caught along Pipeline Road near Gamboa, Panama, in the Soberania National Park, and used to establish stocks at the Smithsonian Tropical Research Institute insectaries in Gamboa. Butterflies were reared in common garden conditions, in 2 × 2 × 2 m cages, and provided with fresh *Psiguria* flowers and 10% sugar solution. Larvae were reared on fresh *Passiflora* shoots/leaves until pupation. *H. cydno*, *H. melpomene* and hybrid individuals used for RNA-seq (see below) were reared concurrently and under the same conditions. F1 hybrids were obtained by crossing a wild-caught *H. m. rosina* male to an insectary-bred virgin *H. c. chioneus* female.

Third-generation backcross hybrids (BC3) were generated by outcrossing a hybrid male with a red forewing band (crossing design shown in Supplementary Fig. 3) to virgin *H. cydno* females, over three generations. The peak of the behavioural QTL reported previously¹⁹ on chromosome 18 (at 0 cM) is in very tight linkage with the *optix* colour pattern locus (at 1.2 cM), which controls for the presence and absence of the red forewing band seen in *H. melpomene rosina*. The presence of the red forewing band is dominant over its absence so that segregation of the red band can be used to infer genotype at the *optix* locus. Specifically, hybrid individuals with a red forewing band are heterozygotes for *H. melpomene/H. cydno* alleles at the *optix* locus, whereas individuals lacking the red band are homozygous for the *H. cydno* allele. Due to the tight linkage we expected little recombination between *optix* and QTL peak even after three generations of introgression, allowing us to infer genotype at the preference-*optix* locus (which we confirmed with genetic data, see below).

Tissue dissection, RNA extraction and mRNA sequencing. Eye (ommatidia and retinal membrane) and brain tissues (central brain and optic lobes) were dissected out of the head capsule (as a single combined tissue) in cold (4 °C) 0.01 M PBS solution, at two pupal stages: 60 h APF and 156 h APF; and in adults aged 9–13 days in 2013. We sampled adults at around 10 days of age because by this stage males are mature and frequently court females⁶⁹. Adult males and females sampled were sexually naive. We decided to sample at 60 h APF because this is the developmental stage at which *optix* is expressed in the wing, so we hypothesized that it might had also been when *optix* is expressed in the brain. We sampled at 156 h APF as a putative stage halfway through pupal development, and at this stage most of the major neural connections have just been established in the *Heliconius* brain (Stephen Montgomery, unpublished data).

Tissues were stored in RNAlater at 4 °C for 24 h, and subsequently at –20 °C, until RNA extraction. Total RNA was extracted using TRIzol Reagent (Thermo Fisher, Waltham, MA, USA) and a RNeasy Mini kit (Qiagen, Valencia, CA, USA). Samples were treated with DNase I (Ambion, Darmstadt, Germany). Integrity of total RNA was checked either on an agarose gel or using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). RNA concentration was measured on a Nanodrop spectrophotometer. Illumina TruSeq RNA-seq libraries were prepared and sequenced at Edinburgh Genomics (Edinburgh, UK) with 100 bp paired-end reads (in 2014). To avoid lane effects the distribution of the species samples was randomized on the sequencing platform. More detailed information about individuals and sequencing yields can be found in Supplementary Data 1. In 2019, we independently sampled a further five *H. melpomene*, five *H. cydno*, and six F1 hybrids males, of 10 days of age. These F1 hybrids were generated by crossing a wild-caught *H. c. chioneus* male to an insectary-bred virgin *H. m. rosina* female. Briefly, for these, RNA was extracted similarly with Trizol Reagent and a PureLink RNA Mini Kit, with PureLink DNase digestion on column (Thermo Fisher, Waltham, MA, USA). Illumina 150 bp paired-end RNA-seq libraries were prepared and sequenced at Novogene (Hong Kong, China).

RNA-seq read mapping and differential gene-expression analyses. After a quality control of RNA-seq reads with FastQC, we trimmed adaptor and

low-quality bases using TrimGalore v.0.4.4 (<https://www.bioinformatics.babraham.ac.uk/projects/>). RNA-seq reads were mapped to the *H. melpomene* 2.5 genome assembly²⁹/annotation³¹ using STAR v.2.4.2a⁷⁰ in two-pass mode. We only kept reads that mapped in ‘proper pairs’ using Samtools⁷¹. The number of reads mapping to each gene was estimated with HTseq v. 0.9.1⁷² with model “union”, thus excluding ambiguously mapped reads. Differential gene-expression analyses between species/hybrids were conducted in DESeq2⁷³, including sequencing batch as random factor when comparing species samples from both datasets. We considered only those genes showing a twofold change in expression level, and at adjusted (false discovery rate 5%) *p* values < 0.05, to be differentially expressed, to exclude expression differences caused by known differences in brain morphology⁷⁴, that in this species pair are clustered in the visual system⁷⁵, although there is no evidence that visual mate preference is linked to these divergent brain morphologies.

Sexing pupae. In all DESeq2 analyses, sex was included as a random factor. To sex pupae, we first marked duplicate RNA mapped reads with Picard (<https://broadinstitute.github.io/picard/>), and used GATK 3.8⁷⁶ to split uniquely mapped reads into exon segments and trim sequences overhanging the intronic regions. We then used HaplotypeCaller on each individual, using calling and filtering parameters according to the GATK Best Practices for variant calling on RNA-seq data. The sex of pupal samples was inferred from the proportion of heterozygous (biallelic) SNPs using the R package SNPstats. Males (ZZ) were expected to have > 0% heterozygous sites, whereas females (ZW) to have 0%. Z-linked heterozygosity of the pupal samples (Supplementary table 4) were in line with expectations (either ~0 for females or an order of magnitude higher for males), and matched heterozygosity of either adult males or females, for which the sex was determined from external morphology.

Inference of gene function and transcript-based annotation. Biological functions of annotated genes were inferred with InterProScan v5⁷⁷, using the corresponding Hmcl2.5 predicted protein sequences. InterProScan uses different databases like InterPro, Pfam, PANTHER, and others, to infer functional protein domains and motifs (based on homology). To study whether specific biological functions were enriched among genes showing differential expression among hybrid types, we conducted the PANTHER enrichment test³⁴ (with Bonferroni correction for multiple testing) using *Drosophila melanogaster* as the reference gene function database.

Upon detailed inspection of the mapping coverage of spliced RNA-seq reads to the Hmcl2.5 gene annotation, we noticed that some gene models were fragmented, namely, a few exons that appeared to be spliced together were incorrectly considered distinct genes. To check that this did not introduced inaccuracies in our differential gene-expression analyses, we re-annotated the *melpomene* genome using the Cufflinks reference annotation-based transcript (RABT) assembly tool³². We used the transcriptomic data from both *H. melpomene* and *H. cydno* to reannotate the *melpomene* genome, separately for every developmental stage, and re-conducted the differential gene-expression analyses in DESeq2 as described above. Repeating all comparative transcriptomic analyses using these new annotations (where exons were correctly considered as part of single genes), we confirmed that both *regucalcin2* and *Grik2* were differentially expressed in both species and hybrids comparisons.

Inference of BC3 hybrids genome composition. In order to perform comparative transcriptomic analyses between third-generation backcross hybrids (BC3) segregating at the QTL on chromosome 18 (crossing design in Supplementary Fig. 3), we first determined which genomic regions in these hybrids were heterozygous (*cyd/melp*) or homozygous (*cyd/cyd*). For this, we inferred variants from RNA-seq reads for each BC3 hybrid (individually as above), and from the combined *H. melpomene* and *H. cydno* samples. For the species, we used HaplotypeCaller⁷⁶ on RNA-seq samples from all developmental stages of either species, to produce individual genomic records (gVCF), and then jointly genotyped *H. melpomene* and *H. cydno* gVCFs (separately for the two species) using genotypeGVCFs with default parameters. Genotype calls were filtered for quality by depth (QD) > 2, strand bias (FS) < 30 and allele depth (DP) > 4. For further analyses we kept biallelic genotypes only. We then used the *intersect* function of *bcftools*⁷¹ to infer variants exclusive to the *H. cydno* and to the *H. melpomene* samples.

We calculated the fraction of variants that each BC3 hybrid individual shared with the *H. melpomene* and with the *H. cydno* samples, in non-overlapping 100 kb windows. We compared these to the fraction of variants that a F1 hybrid and a *H. cydno* individual (not included in the combined genotyping of the *H. cydno* samples), shared with the same species samples, and found that they matched either one of them, indicating heterozygous (*cyd/melp*) or homozygous (*cyd/cyd*) regions (Supplementary Fig. 5B). In this analysis, we considered only those 100 kb windows where BC3 hybrids/F1 hybrid/*H. cydno* individuals shared more than 30 variants with the *melpomene/cydno* samples.

To corroborate our findings, we repeated the same type of analysis, this time inferring species-specific variants for *H. melpomene* and *H. cydno* using 10 *H. melpomene rosina* and 10 *H. cydno chioneus* genome resequencing samples.

Variant calling files (vcf) were retrieved from Martin et al.³⁷ We considered only biallelic genotype calls that had 10 < DP < 100 and genotype quality > 30. With this analysis we found the same heterozygous and homozygous regions in BC3 hybrids.

The size and number of the introgressed regions were in line with expectations about 3rd generation backcross hybrids following our crossing design: segregating at the level of chromosome 18 and at four other chromosomes. For the BC3 hybrids sampled at 156 h APF we had 6 *cyd/melp* and 10 *cyd/cyd* at the QTL region on chromosome 18 (Supplementary Fig. 5A), for those at 60 h APF, 8 *cyd/melp* and 9 *cyd/cyd* hybrids at the same region. The average percentage of the genome that is heterozygous (*cyd/melp*) as opposed to homozygous (*cyd/cyd*) outside of chromosome 18 was ~6% (close to the expectation that a 3rd generation backcross genome should be 1/16 heterozygous (*cyd/melp*)).

Allele specific expression in hybrids. In order to conduct allele specific expression analyses we first identified species-specific variants, fixed in either *H. melpomene* and *H. cydno*. For this, we took the quality filtered variants inferred from the species genome resequencing data, and assigned those genotype calls in *H. cydno* and *H. melpomene* for which allele frequency (AF) was > 0.9 as homozygous (we did not consider indels in this analysis). We then used *bcftools intersect*⁷¹ to get only those variants for which *H. cydno* and *H. melpomene* had opposite alleles.

At the same time, we called variants from RNA-seq reads of F1 hybrid individuals (using F1 hybrids from both datasets), again according to the GATK Best Practices (with the exception of parameters -window 35 -cluster 3, to increase SNPs density), and selected only heterozygous SNPs in F1s that matched the species-specific variants. Finally, we used GATK’s ASEReadCounter⁷⁶, with option “-drf DuplicateRead” (without deduplicating RNA reads), to count RNA reads in the F1 hybrids (and later on in BC3 hybrids) that mapped to either the *H. cydno* or the *H. melpomene* allele. We summed all reads mapping to either the *H. cydno* or *H. melpomene* allele/variant within the same gene (both for gene models of the Hmcl2.5 gene annotation and for the Cufflinks annotation we assembled previously). To test for allele-specific expression (diffASE) we fitted the model “~0 + individual + allele” in DESeq2⁷³, setting library size factors to 1 (thus not normalizing between samples, as the test for diffASE is conducted within individuals). We only considered those alleles showing at least a twofold change in expression and *p* < 0.05, as differentially expressed.

In order to check that there were no biases in alleles assignment to one of the two species, we analyzed the ratios of the species alleles, for every gene, and checked that they were not systematically biased to either one of the two species. The log₂ fold changes of the species alleles were centred around 0, suggesting no obvious bias in alleles assignment⁷⁸ (Supplementary Fig. 7).

Protein-coding substitutions and predicted effects on protein-function. We inferred fixed variants in protein-coding regions from the combined *H. melpomene* and *H. cydno* RNA samples in order to include variants from genes for which we detected expression in the brain/eyes across the 3 stages. We took the quality filtered variants called from the joint genotyping of RNA-seq data of *H. cydno* and *H. melpomene* (from all stages, these did not include adult samples from the more recent sequencing batch), and selected those genotype calls for which AF > 0.8, and where the allelic variant was present in at least 7 individuals of the ~30 samples (for each species). We retained those substitutions/indels validated with the genome resequencing data. For this, of the genotype calls found in RNA reads from brain/eyes of different stages, we kept only those that were also called in at least 8 of the 10 genome resequencing samples of each species. We considered this overlapping set of variants as being fixed in *H. melpomene rosina* or *H. cydno chioneus*. Following a similar approach to Bendesky et al.⁶⁶, we then restricted this set of substitutions between *H. cydno* and *H. melpomene* to protein-coding regions, and selected those non-synonymous substitutions that were considered to have moderate or high effect on protein function with SnpEff⁷⁹. Finally, we used the PROVEAN algorithm³⁵, to further study the functional effects of these substitutions on protein function. The PROVEAN algorithm predicts the functional effect of protein sequence variations based on how they affect alignments to homologous protein sequences (for this we used the PROVEAN protein database online). We selected those amino acid changes with the suggested PROVEAN score < -2.5, indicating non-neutral effect on protein function.

Admixture analyses. We retrieved estimated admixture proportions between *H. melpomene rosina* and *H. cydno chioneus*, for 100 kb and 20 kb windows, from Martin et al.³⁷.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

RNA-seq data (raw reads) have been deposited in the European Nucleotide Archive (ENA) (accession number: PRJEB39935). Individual samples’ accession numbers and detailed metadata are reported in Supplementary Data 1. Previously published genomic data are available at: [<https://www.ebi.ac.uk/ena/browser/view/PRJEB1749>] and at [<https://www.ncbi.nlm.nih.gov/bioproject/PRJEB11772>].

Code availability

Analysis scripts and behavioural data are available at: https://github.com/SpeciationBehaviour/neural_genes_heliconius.git

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Author contributions

R.M.M. and M.R. conceived the study and designed the experiments, with input from W.O.M. and C.D.J.; M.R. analysed expression and sequence data; A.E.H. analysed the behavioural data; M.R., T.J.T., S.H.M. and R.M.M. reared butterflies, dissected neural tissue and extracted RNA; R.M.M., C.D.J. and W.O.M. secured funding, contributed resources and provided supervision; R.P. additionally secured funding and contributed resources; M.R. and R.M.M. wrote the manuscript with contributions from all authors.

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