

Published in final edited form as:

*Science*. 2024 March 22; 383(6689): 1368–1373. doi:10.1126/science.adj9201.

## Adaptive introgression of a visual preference gene

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### Abstract

Visual preferences are important drivers of mate choice and sexual selection, but little is known of how they evolve at the genetic level. We take advantage of the diversity of bright warning patterns displayed by *Heliconius* butterflies, which are also used during mate choice. Combining behavioral, population genomic and expression analyses, we show that two *Heliconius* species have evolved the same preferences for red patterns by exchanging genetic material through hybridization. Neural expression of *regucalcin1* correlates with visual preference across populations, and disruption of *regucalcin1* with CRISPR/Cas9 impairs courtship towards conspecific females, providing a direct link between gene and behavior. Our results support a role for hybridization during behavioral evolution, and show how visually-guided behaviors contributing to adaptation and speciation are encoded within the genome.

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Organisms often use color, and other visual cues, to attract and recognize suitable mates (1). The evolution of these cues is increasingly understood at the molecular level,

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Visualization: MR, AEH, PA, RMM

Funding acquisition: RMM

Project administration: ML, WOM, CP-D, CS, RMM

Supervision: PA, WOM, CO, CP-D, CS, RMM

Writing – original draft: MR, RMM

Writing – review & editing: All authors

**Competing interests:** Authors declare that they have no competing interests.

providing insights into the nature and origin of genetic variation on which selection acts e.g., (2–7). However, we know little of the genetic mechanisms underlying variation in the corresponding preferences, or visually guided behaviors more broadly. Indeed, while progress has been made for other sensory modalities, and especially chemosensation, e.g., (8–10), genetic studies of visual preference evolution remain limited to the identification of relatively broad genomic regions containing tens or hundreds of genes, and/or are unable to distinguish between causal and correlated genetic changes (11–15). Although these studies have undoubtedly contributed to our understanding of population divergence, identifying the causal genes involved is key to uncovering how behavioral variation is generated during development and across evolutionary time.

*Heliconius* butterflies are well known for their diversity of bright warning patterns, which are also used as mating cues (16), perhaps alongside olfactory cues (17). Closely related taxa often display divergent wing patterns, and because males almost invariably prefer to court females that share their own color pattern, this contributes an important premating reproductive barrier between species e.g., (18). While the genetics and evolutionary history of *Heliconius* color pattern variation is well understood (19–25), we know little of the specific genetic mechanisms contributing to the evolution of the corresponding visual preference behaviors. Previously, we identified three genomic regions controlling differences in male courtship behaviors between the closely-related sympatric species *H. cydno* and *H. melpomene*, which differ in color pattern (11). However, further fine mapping of this behavioral phenotype is impractical, and even the best supported of these behavioral quantitative trait loci (QTLs), which has also been explicitly linked to differences in visual preference (26), is associated with a confidence region containing 200 genes. Although patterns of neural gene expression highlight a number of candidates (27), the exact genes involved remain unknown.

Here we take advantage of the mimicry relationships among three closely related *Heliconius* species to determine how genetic variation for visual preferences has evolved in relation to that of the corresponding color pattern cues. Whereas west of the Eastern Cordillera in the Andes coexisting *H. cydno* and *H. melpomene* differ in forewing color (being white and red respectively), on the eastern slopes *H. cydno* is replaced by its sister species *H. timareta*, which shares the red patterns of the local *H. melpomene* (Fig. 1A). Mimicry between these two red species is not the result of independent mutations, but adaptive introgression, whereby *H. timareta* acquired color pattern alleles following hybridization with *H. melpomene* (24, 25, 28). This presents an excellent opportunity to both i) test whether behavioral phenotypes can similarly evolve through the reassembly of existing genetic variants on a novel genomic background, and ii) to isolate the causal genes. We identify a region of increased admixture between *H. melpomene* and *H. timareta* that is strongly associated with parallel preferences for red females in both species. We then leverage this finding alongside transcriptomic analysis and genome-editing to identify a major effect gene underlying the evolution of visual preferences.

## Evolution of parallel visual preference behaviors

To explore the evolution of visually guided behaviors across the *melpomene-cydno* group we assayed mate preference for populations sampled across Colombia. Specifically, we tested *H. melpomene* and *H. timareta* males from the eastern slopes of the Eastern Cordillera, which both have a red forewing band, as well as *H. cydno* males from the western slopes of the Eastern Cordillera, which have a white or yellow forewing band. Male butterflies were simultaneously presented with a red *H. timareta* and a white *H. cydno* female in standardized trials. Males of the two red species showed a stronger preference for red females than the *H. cydno* males (differences in proportion courtship time towards red females: *H. timareta* - *H. cydno* = 0.737 [0.630 – 0.844], *H. melpomene* - *H. cydno* = 0.713 [0.593 – 0.832];  $n = 87$ ,  $2 \ln L = 99.8$ ,  $P \ll 0.0001$ ; Fig. 1B), but there was no difference in mate preference between the two red species (0.025 [-0.039 – 0.087]). We confirmed that preference differences between male *H. timareta* and *H. cydno* are largely based on visual cues by repeating our experiment, this time presenting males with two *H. cydno* females, where the forewings of one were artificially colored to match the red forewing of *H. timareta* (with respect to *Heliconius* color vision), and the wings of the other with a transparent marker as a control (*H. timareta* - *H. cydno* = 0.46 [0.36 – 0.56];  $n = 94$ ,  $2 \ln L = 53.7$ ,  $P \ll 0.0001$ , Fig S1). Overall, these results closely mirror previous data for Panamanian populations of *H. cydno* and *H. melpomene* (11, 18), where the latter shows a much stronger preference for red females, and confirms that although *H. timareta* is more closely related to *H. cydno*, it shares the visual preference phenotype of *H. melpomene*.

## The same major effect locus contributes to red preference in *H. melpomene* and *H. timareta*

If introgression has contributed to these parallel behavioral preferences for females with red patterns, we would expect the same genomic locations to influence the preference behaviors of both *H. melpomene* and *H. timareta*. In other words, we expect that the alleles at the location of the *H. melpomene* x *H. cydno* QTL to also segregate with preference differences in crosses between *H. timareta* and *H. cydno*. Confirming this, we found that genotype at the end of chromosome 18 is a strong predictor of male preference in *H. timareta* x *H. cydno* hybrids. Specifically, backcross hybrid males that inherit an allele from *H. timareta* at the previously detected QTL peak spent more time courting red *H. timareta* than white *H. cydno* females, compared to their brothers that inherited two copies of the *H. cydno* alleles at the same location (differences in proportion courtship time between males with ‘cydno-timareta’ and ‘cydno-cydno’ genotypes = 0.279 [0.137 – 0.42];  $n = 157$ ,  $2 \ln L = 14.02$ ,  $P = 0.00018$ ; Figs. 1C and D). Notably the effect size observed here is almost identical to that seen in hybrids between *H. cydno* and *H. melpomene* (i.e., 0.249 [0.168 – 0.33]; Fig. 1E).

To further confirm that the QTL region on chromosome 18 specifically modulates visual mate preferences, we also assayed mate preference behaviors of *H. timareta* x *H. cydno* hybrid males towards white (transparently-painted) and red-painted *H. cydno* females (as described above). We found that backcross males heterozygous for *H. timareta* and *H. cydno* alleles at the QTL confidence region on chromosome 18 court red-painted females

more frequently than their brothers homozygous for the *H. cydno* allele ( $n = 270$ ,  $2 \ln L = 7.811$ ,  $P = 0.005$ , Fig. S1). While the effect size for this experiment (0.0778 [0.024 – 0.13]) is reduced compared to that seen for experiments using *H. timareta* females, this still represents a considerable proportion of the observed parental difference (~17%). Together our two experiments confirm that the same genomic region at the end of chromosome 18 modulates variation in visual mate preferences across the *melpomene-cydno* group.

## Genomic signatures of adaptive introgression at the preference locus

To further determine whether introgression of preference alleles has contributed to behavioral evolution in these species, we next analyzed admixture proportions ( $f_d$ , (29)) between sympatric red-preferring *H. melpomene* and *H. timareta*. We observed two striking peaks of admixture in the QTL region on chromosome 18, located within the behavioral QTL peak, i.e., the region of greatest statistical association with difference in male preference between *H. cydno* and *H. melpomene*, and upstream of the adjacent major color pattern gene, *optix*, corresponding to its putative regulatory region (5, 30) (Fig. 2, c.f. Fig. S2). Admixture estimates are repeatable across geographic populations of *H. melpomene* and *H. timareta*, and are independent of variation in local recombination rates, known to otherwise correlate with admixture proportions (31) (Fig. 2).

Introgression at the two loci on chromosome 18 is further supported by analyses using *Twisst* (32), which quantifies the proportion of different phylogenetic relationships among individuals of different species across the chromosome. In these analyses, the “introgression” topology, where *H. timareta* and *H. melpomene* cluster together, with *H. cydno* as an outgroup, is strongly supported both within the QTL peak and at *optix* (Figs. 2 and S3). These admixture peaks of approximately 30kb and 150kb, respectively, additionally coincide with elevated levels of genetic differentiation ( $F_{ST}$ ) and absolute genetic divergence ( $d_{xy}$ ) between red- and white-preferring populations (Fig. 2). Patterns of linkage disequilibrium between these two loci are consistent with the genetic associations predicted to arise between cue and preference alleles as a result of assortative mating (8, 33) (Fig. S4). Finally, using *Sweepfinder2* (34), we found evidence for a recent selective sweep in *H. timareta* (top 1% quantile across autosomes), coincident with the peak of increased admixture within the behavioral QTL peak described above, but not at *optix* (Figs. 2 and S5). These results suggest adaptive introgression of alleles from red-preferring *H. melpomene* into *H. timareta* at a genomic location strongly associated with variation in visual preference.

## Cis-regulated expression differences of *regucalcin1* are associated with visual preference

We next generated RNAseq libraries for combined eye and brain tissue from adult males across all populations tested in our preference assays to determine whether consistent differences in gene expression are associated with the behavioral QTL on chromosome 18. We sampled at the adult stage reasoning that if the neural mechanism underlying divergent preference behaviors involves a change in neuronal activity, this might require sustained transcription. Of 200 genes within the chromosome 18 QTL candidate region,

although a number were differentially expressed in individual comparisons (Fig. S6), only one was consistently differentially expressed across all red and white preferring population comparisons (reared under common garden conditions, Fig. S7). Specifically, *regucalcin1*, which perfectly coincides with the peak of adaptive introgression between red-preferring populations detected above, shows lower expression in the neural tissue of Panamanian and Colombian populations of *H. melpomene* and *H. timareta*, all of which we have shown to have a red preference as compared to *H. cydno* (Figs. 3A and S7). Expression of *regucalcin1* is also significantly reduced in *H. melpomene amaryllis* and *H. melpomene melpomene* as compared to *H. cydno*, two populations also known to display a preference for red females (18, 35) (Fig. S7). Immunostainings in adult male *H. melpomene* revealed expression patterns of *regucalcin1* in the visual pathways across the brain, predominantly in somata, especially the nuclei, and also in neuropil, as well as in the eye (Fig. 3C and S8). Although this does not pinpoint the particular mechanism of action, it confirms that regulatory changes of *regucalcin1* have the potential to affect visual preference behavior.

If expression differences in *regucalcin1* are responsible for the behavioral variation associated with the QTL on chromosome 18, they must result from changes within the *cis*-regulatory regions of the genes themselves, as opposed to those of other *trans*-acting genes elsewhere in the genome. To test whether differences in gene-expression levels between parental species were due to *cis*- or *trans*-regulatory changes, we conducted allele-specific expression analyses in adult male F1 *H. melpomene* x *H. cydno* and *H. cydno* x *H. timareta* hybrids. 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). Confirming *cis*-regulation of *regucalcin1*, we found a significant 2-fold up-regulation of the *H. cydno* allele relative to the *H. melpomene* or *H. timareta* allele in the neural tissue of both our *H. melpomene* x *H. cydno* and *H. timareta* x *H. cydno* F1 males (Wald test all comparisons:  $P < 0.001$ , Fig. 3B).

## CRISPR/Cas9 mediated knock-out of *regucalcin1* disrupts male courtship behaviors

Combining genetic crosses and behavioral data, as well as population genomic and expression analyses, our results strongly implicate *regucalcin1* as a visual preference gene. To functionally test for a link between gene and behavior, we knocked-out the protein coding region of *regucalcin1* in *H. melpomene* individuals by introducing a ~1300bp deletion spanning most of its first and second exon using CRISPR/Cas9 (Fig. 4A). In trials with a single conspecific female (Fig. 4B), mosaic knock-out (mKO) males (i.e., those with a deletion at *regucalcin1* in a substantial number of cells, including in brain tissue, Fig. S9) were significantly less likely to court than control (ND) males without the deletion (difference in proportion minutes courting, trials with mKO males - trials with ND males = 0.24 [0.03-0.55]; 2  $\ln L = 4.51$ ,  $P < 0.05$ ; Fig. 4C). mKO knockout individuals may suffer decreased viability both pre- and post-eclosion (Fig. S10), and some mKO butterflies were unable to fly (8/44 individuals) as determined in our 'drop test' (as compared to 0/40 ND individuals or 0/42 wildtype individuals; Fisher exact test:  $P < 0.001$ ). However, only

surviving males that could fly were included in our courtship trials. Furthermore, all mKO (36/36), ND (31/31) and wildtype (30/30) individuals tested, including seven individuals that failed the subsequent drop test, showed an optomotor response (Movie S1), suggesting basic visual sensorimotor skills are largely intact in mKO individuals. Finally, we observed no difference in the proportion of time flying or feeding between the same mKO or ND males included in our courtship trials (0.01 [-0.07-0.097]; 2  $\ln L = 0$ ,  $P > 0.9$ ; Fig. 4C and S11). In other words, courtship – but not other behaviors – was significantly reduced in *regucalcin1* knockout males as compared to controls, which retain functional copies of *regucalcin1*. This provides functional evidence that *regucalcin1* has a specific effect on male courtship behavior, and that this is not due to a more general impairment of behavior.

## Conclusions

Hybridization has been suggested to be an important source of genetic variation on which selection can act, including during behavioral evolution (36, 37), but direct links between specific causal genes and behavioral phenotypes are lacking. Our results strongly suggest that *Heliconius timareta* acquired a *regucalcin1* allele by hybridizing with its closely related co-mimic *H. melpomene*, increasing attraction towards red females, and presumably reproductive success. In contrast, where *H. melpomene* co-occurs with the equally closely related but differently colored *H. cydno*, *regucalcin1* contributes an important barrier to interspecific gene flow through its contribution to divergent mating preferences (11, 38). As such, the evolutionary impact of *regucalcin1* depends on the local mimetic landscape, emphasizing the complex role that hybridization may have on population divergence by reassembling genetic variants (39).

Although other genes aside from *regucalcin1* undoubtedly contribute to visual preference evolution in *Heliconius*, there is little evidence that these include major wing-patterning genes. There is no evidence for differential expression of *optix* between red and white preferring populations, and protein coding differences similarly do not exist (27). Instead, our data suggest that although variation in red color cue and preference map to the same genomic region, they are encoded by separate loci regulating the expression of *optix* (19) and *regucalcin1*, respectively (Fig. S12). By ensuring robust genetic associations between components of reproductive isolation, physical linkage is expected to facilitate speciation with gene flow (40), and this is likely the case for the differently colored species *H. cydno* and *H. melpomene* (11). However, our present results suggest these loci can also evolve independently, and evidence of a recent selective sweep in *H. timareta* at *regucalcin1*, but not *optix*, as well as distinct peaks of admixture between red-preferring species at these two genes, suggest separate introgression events. It seems likely that the acquisition of red patterns in *H. timareta* was immediately advantageous given strong selection for mimicry of local warning patterns, whereas the corresponding male preference would become advantageous only when conspecific red females had already increased in frequency.

Other prominent examples of visual preference evolution have emphasized the role of selection imposed by the broader sensory environment. In cichlid fish, for example, divergent mating preferences may have evolved as a by-product of environmental selection acting on visual pigment genes (15, 41). Interestingly, *H. timareta* and *H. melpomene*



have evolved parallel visual preferences despite inhabiting divergent light environments (*H. timareta* is found in similar forest habitats to *H. cydno*), to which the neural and sensory systems are otherwise adapted (42). This suggests that visual preference evolution in *Heliconius* is not the by-product of divergent selection imposed by the broader sensory environment, but rather a consequence of direct selection to find receptive females, perhaps strengthened through reinforcement (where selection favors increased premating barriers to avoid the production of less fit hybrids) (18, 43, 44).

Overall, our study indicates that the evolution of *cis*-regulated differences in *regucalcin1* expression contributes to divergent mating preferences in *Heliconius*, and that hybridization can be an important source of genetic variation during behavioral evolution. The function of *regucalcin* has not been well characterized though it seems to be involved in calcium homeostasis and signaling (45). Our CRISPR-mediated *regucalcin1* knock-out impaired survival and flight in a few mosaic butterflies, supporting a broad role across biological processes. However, in other mosaic knock-out individuals we observed a significant reduction in mate attraction behaviors, independent of more general impairment of motor activity, implying specific effects on male mating behavior. *Regucalcin1* expression differences, sustained in adult brain and/or eye tissue, likely alter how visual information is processed or integrated to determine divergent mating preferences. The challenge now is to determine the molecular and neural mechanisms through which it acts.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We dedicate this paper to the memory of our friend and colleague Alexander E. Hausmann. We are grateful to Bianca Hoelldobler, Isabel Leon, Francesco Rossi, Rebecca Stephens, Sophie Smith, José Borrero, Marilia Freire, Alberto Comin, Christina Burrows, Michaela Bauer, Yi-Peng Toh and Christine Rottenberger for technical and rearing assistance, Benedikt Grothe for providing research infrastructure, and Mathieu Choteau for help with fieldwork. We thank Francesco Cicconardi for sharing a pipeline for ISO-Seq analysis and Simon Martin for sharing vcf files. We thank Philipp Brand, Max Farnworth, Nicolas Gompel, Joseph Hanly, Luca Livraghi, Ricardo Pereira, Jochen Wolf, Stephen Montgomery and Vera Warmuth for valuable input on methods and the manuscript. We thank the Universidad del Rosario and the Smithsonian Tropical Research Institute for providing butterfly maintenance and rearing support in Colombia and Panama, respectively. We are grateful to *Autoridad Nacional de Licencias Ambientales*, Colombia (permit 530) and the *Ministerio de Ambiente*, Panama (permit SE/AP-14-18) for permission to collect butterflies.

## Funding

Deutsche Forschungsgemeinschaft (DFG) Emmy Noether grant GZ:ME 4845/1-1 (RMM)

ERC Starting grant 851040 (RMM)

## Data and materials availability

Custom scripts, analyses pipelines, and raw data are available through the archived Zenodo repository (47). Whole-genome resequencing, RNA-Seq and ISO-Seq data are available at the European Nucleotide Archive (ENA): <https://www.ebi.ac.uk/ena/browser/view/PRJEB69696>. Previously compiled data were retrieved from ENA with the following

accession numbers: PRJEB39935, PRJEB35570 and PRJEB1749. More information can be found in Supplementary File 1.

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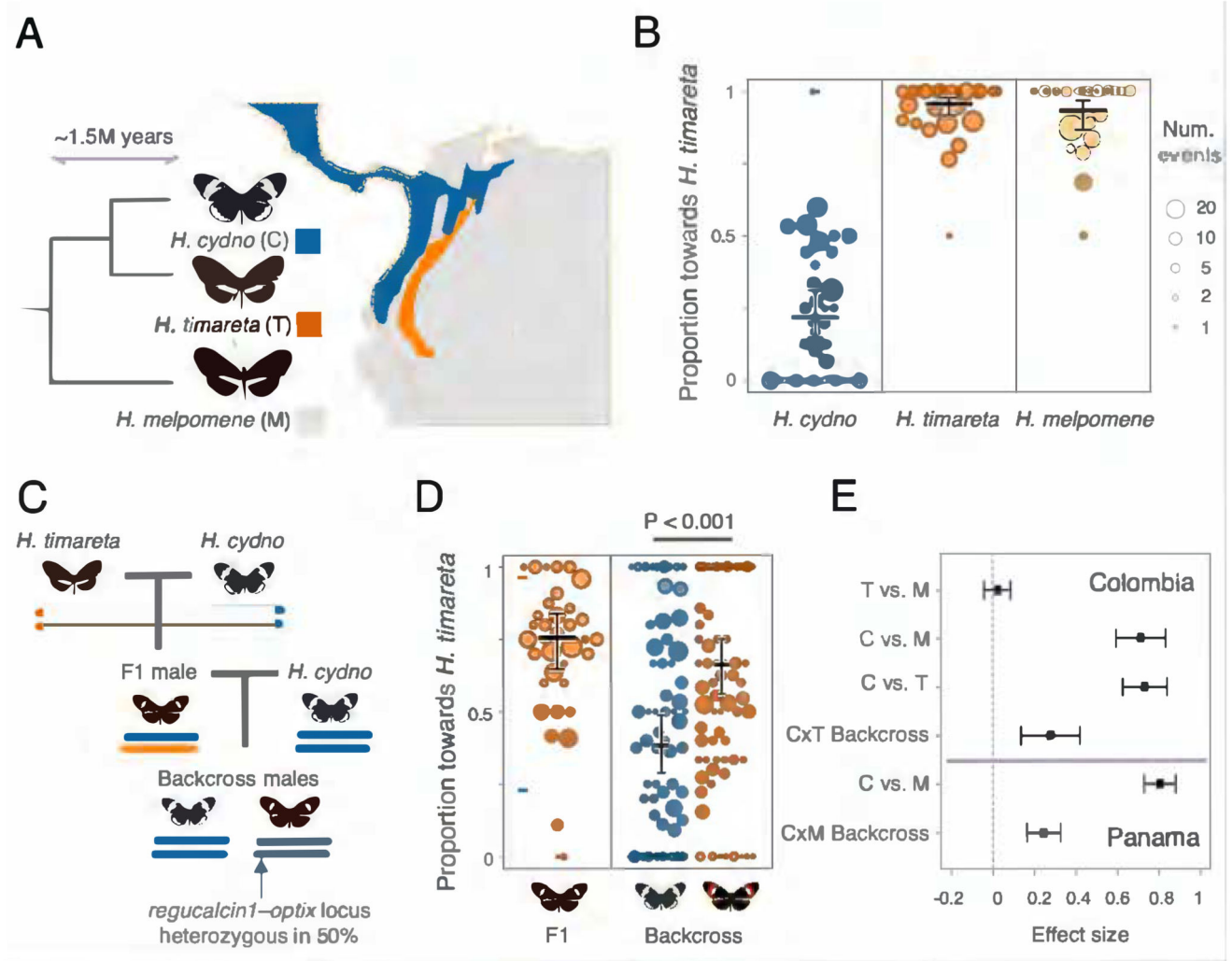
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### One-Sentence Summary

Visual mating behaviors in *Heliconius* butterflies are controlled by *regucalcin1*, which has been shared through hybridization.

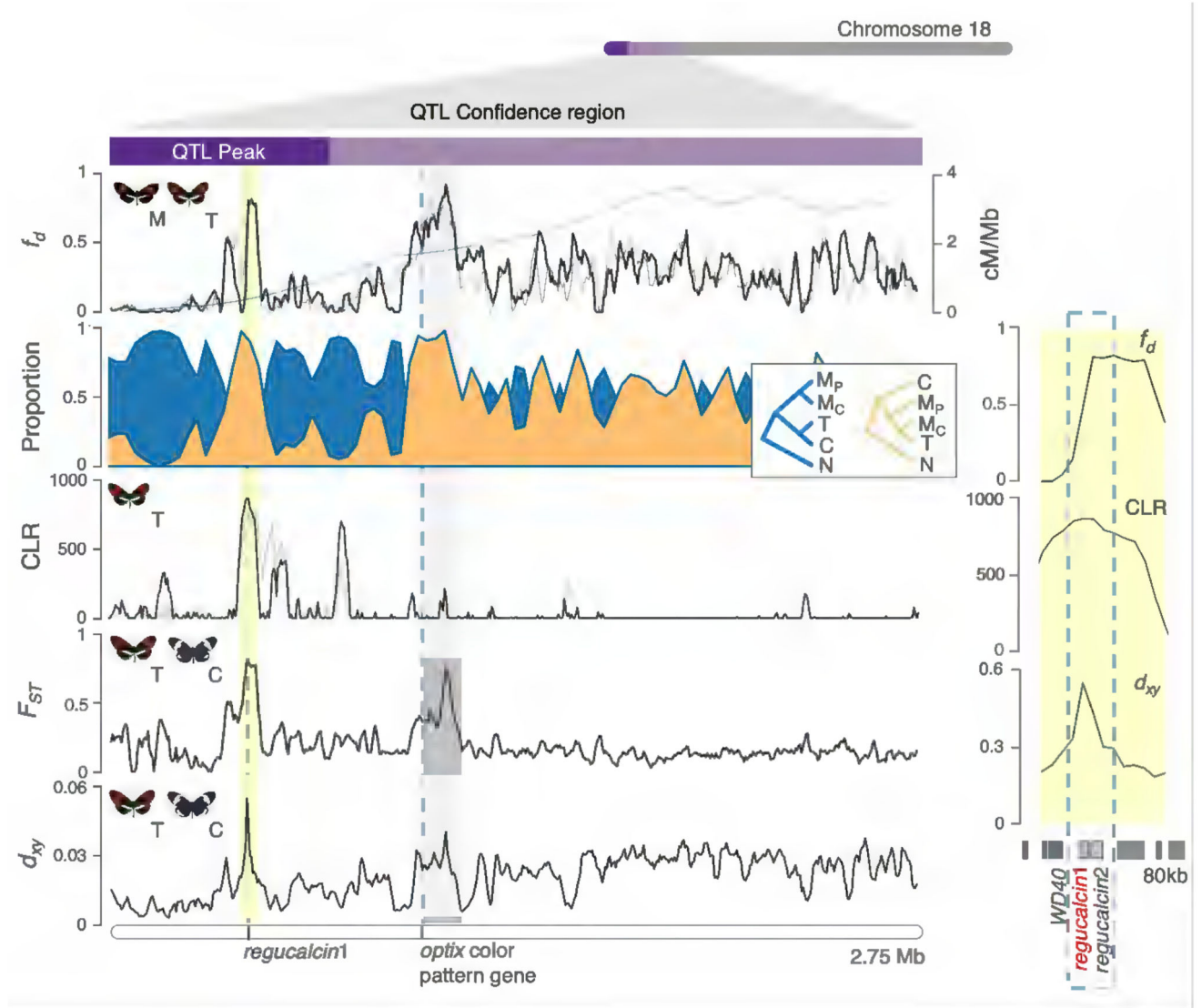


**Fig. 1. Parallel visual preferences are controlled by the same genomic region in the *Heliconius melpomene-cydney* group.**

(A) *H. melpomene* (dotted orange line) co-occurs with *H. cydno* (blue) in Central America and South America to the west of the Eastern Cordillera in the Andes, while *H. melpomene* co-occurs with *H. timareta* (orange) to the east of the Eastern Cordillera. *H. melpomene* and *H. timareta* share red warning patterns even though the latter is more closely related to the white/yellow *H. cydno*. (B) Proportion of courtship time directed towards red *H. timareta* females relative to white *H. cydno* females by males of the three species. Point size is scaled to the number of total minutes a male responded to either female type (a custom swarmplot was used to distribute dots horizontally). Estimated marginal means and their 95% confidence intervals are displayed with black bars. (C) Crossing design for producing backcross hybrid individuals to *H. cydno* segregating at the behavioral QTL region on chromosome 18. (D) Relative courtship time directed towards red *H. timareta* females by F1 hybrid and backcross to *H. cydno* hybrid males. Orange points represent individuals that are heterozygous (i.e., ‘cydno-timareta’) and blue points represent individuals that are homozygous for *H. cydno* alleles at the QTL peak/*optix* region on chromosome 18.



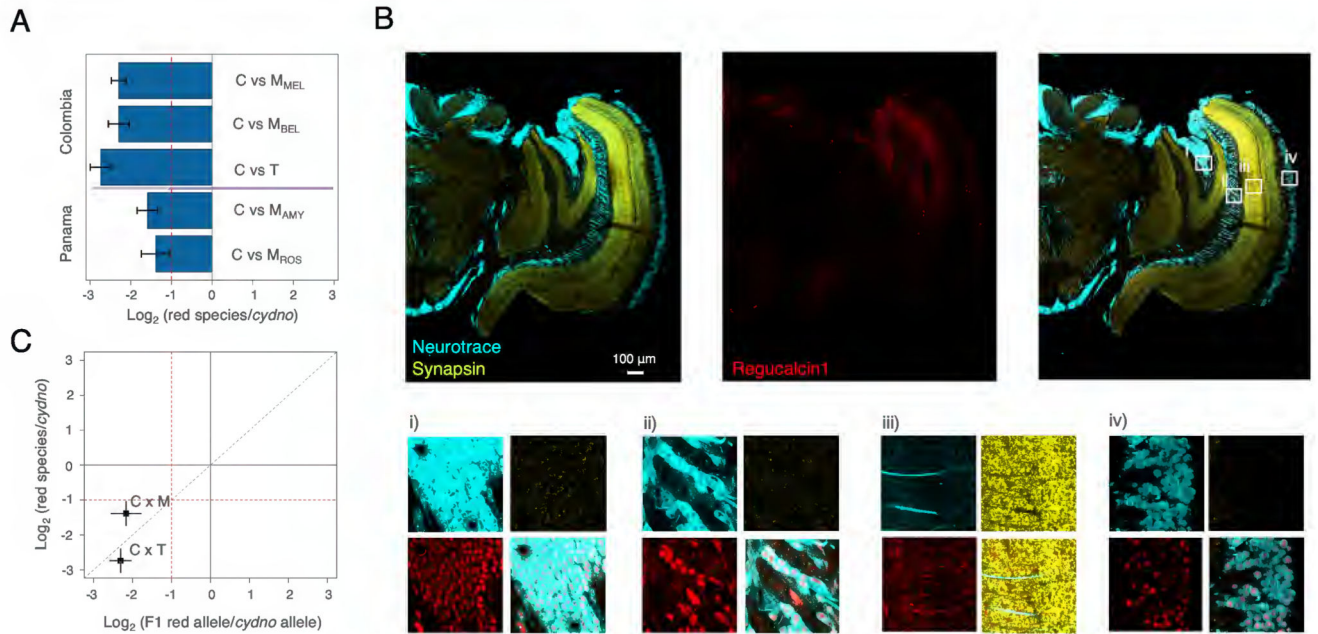
Note that although we observe evidence of recombination in our crosses, the QTL peak/optix region on chromosome 18 often segregates with warning pattern (see supplementary Materials and Methods (46)). **(E)** Differences in estimated marginal means for relative courtship time between butterfly types tested in Colombia (this study) and in Panama (11). T = *H. timareta*, M = *H. melpomene*, C = *H. cydno*, Backcross = backcross to *H. cydno* hybrids.



**Fig. 2. Different genomic signatures support both divergence and adaptive introgression at the *regucalcin* locus.**

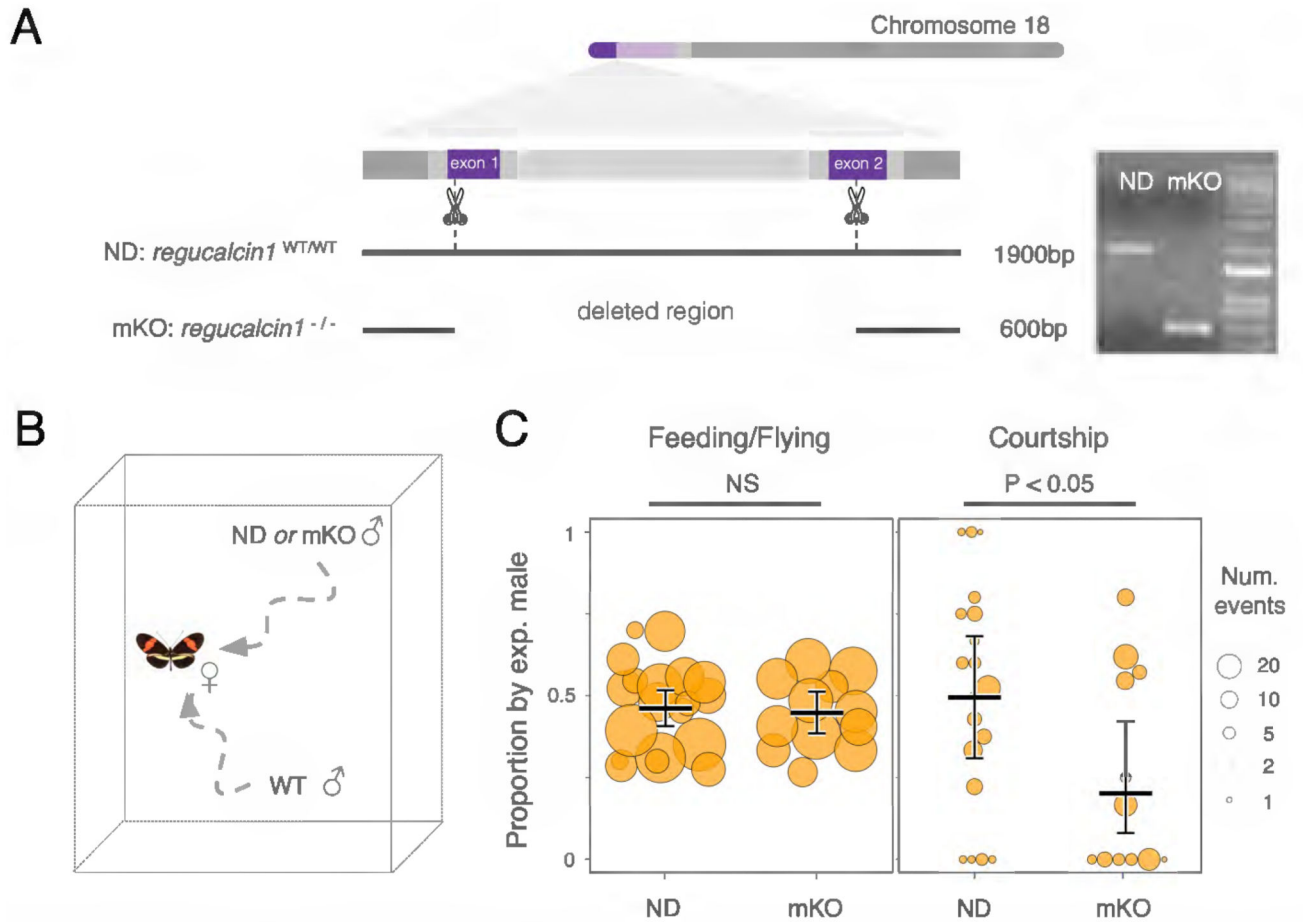
Left, from top to bottom: Admixture proportion values (20kb windows) between *H. melpomene* and *H. timareta* at the behavioral QTL region on chromosome 18 (x-axis indicates physical position) for Colombian (black) and Peruvian (gray) populations, with recombination rate overlaid in blue; topology weightings (proportions of a particular phylogenetic tree over all possible rooted trees) for the “species” (blue) and “introgression” (orange) trees (50 SNPs windows, a *loess* smoothing function across 150kb windows was applied). *H. numata* was used as outgroup; composite likelihood ratio (CLR) of a selective sweep in *H. timareta* (50 SNPs windows); fixation index ( $F_{ST}$ ) and  $d_{xy}$ , measures of genetic differentiation and divergence, between *H. timareta* and *H. cydno*. The gene coordinates of the candidate gene for behavioral difference *regucalcin1* as well as the color pattern gene *optix* (~550 kb apart) are highlighted by vertical light blue dotted lines, and the putative regulatory regions of *optix* affecting color pattern are indicated by gray shading. Note that

the QTL confidence region contains 200 genes (47). Panel to the right zooms into the region containing candidate behavioral genes. M, T, C and N denote *H. melpomene*, *H. timareta*, *H. cydno* and *H. numata*, respectively; subscripts <sub>P</sub> and <sub>C</sub> denote Panama and Colombia, respectively.



**Fig. 3. Cis-regulated expression differences of *regucalcin1* are associated with visual preference and *regucalcin1* is expressed in the visual pathways.**

(A) *Regucalcin1* is differentially expressed between red-preferring and white-preferring butterflies. Histogram heights represent the value and bars the standard error of the (base 2) logarithmic fold change in expression between red-preferring and white-preferring *Heliconius* subspecies (comparisons conducted only between butterflies raised in the same insectary locations). The dashed red line indicates the threshold for a 2-fold change in mRNA expression. M, T, C denote *H. melpomene*, *H. timareta* and *H. cydno*, respectively; subscripts <sub>p<sub>C</sub></sub> and <sub>p<sub>e</sub></sub> denote Panama, Colombia and Peru, respectively. (B) Allele specific expression analyses indicate that differences in expression of *regucalcin1* in the brains of red and white preferring population are *cis*-regulated. Points indicate the value and bars the standard error of the log<sub>2</sub> (fold change) in expression between parental species (vertical) and the alleles in F1 hybrids (horizontal), for *regucalcin1*. Dashed red lines indicate the threshold for a 2-fold change in expression for the genes in the species (horizontal), and for the alleles in the hybrids (vertical). *Regucalcin1* is largely *cis*-regulated (indicated by proximity to  $y = x$ ). (C) *Regucalcin1* is widely expressed in *Heliconius melpomene* brains, including the visual pathways, and eye (Fig. S8). On top, immunostaining of the right hemisphere, from left to right: counterstaining of somata with *neurotrace* and of the neuropil with *synapsin*, center: staining against *regucalcin1*, right: merged image. Below, enlargement of somata (i, iii, iv), where the signal is particularly strong in nuclei, and neuropil (ii) along the visual pathways.



**Fig. 4. Disruption of *regucalcin1* with CRISPR/Cas9 impairs male courtship behavior.**

(A) Left: schematic representation of the *regucalcin1* locus with the target sites of the small guide RNAs and resulting CRISPR/Cas9-mediated deletion of ~1300bp. Right: gel electrophoresis of PCR-amplified *regucalcin1* fragments from individuals without (ND) and with deletion (mKO) at *regucalcin1*. (B) Schematic representation of courtship trials. Experimental (i.e., a mKO or ND) males that passed our ‘drop test’ were paired with a wildtype (WT) male and introduced into a cage with a wildtype virgin *H. melpomene* female. This paired design allowed us to control for both the injection procedure, as well as prevailing conditions that might potentially influence male behavior. (C) Proportion of time spent flying or feeding by experimental (‘exp’) males, i.e. those injected but non-deletion (ND) males or *regucalcin1* mosaic knock-out (mKO) males, relative to wildtype (WT) males (left panel); proportion of courtship time directed towards the same *H. melpomene* female by injected but non-deletion (ND) males (left) and *regucalcin1* mosaic knock-out (mKO) males relative to wildtype (WT) males (right panel). Point size is scaled to the number of total minutes a male flew/fed or courted during the experiments.