Divergence of chemosensing during the early stages of speciation

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Chemosensory communication is essential to insect biology, playing indispensable roles during mate-finding, foraging, and oviposition behaviors. These traits are particularly important during speciation, where chemical perception may serve to establish species barriers. However, identifying genes associated with such complex behavioral traits remains a significant challenge. Through a combination of transcriptomic and genomic approaches, we characterize the genetic architecture of chemoperception and the role of chemosensing during speciation for a young species pair of *Heliconius* butterflies, *Heliconius melpomene* and *Heliconius cydno*. We provide a detailed description of chemosensory gene-expression profiles as they relate to sensory tissue (antennae, legs, and mouthparts), sex (male and female), and life stage (unmated and mated female butterflies). Our results untangle the potential role of chemical communication in establishing barriers during speciation and identify strong candidate genes for mate and host plant choice behaviors. Of the 252 chemosensory genes, *HmOBP20* (involved in volatile detection) and *HmGr56* (a putative synephrine-related receptor) emerge as strong candidates for divergence in pheromone detection and host plant discrimination, respectively. These two genes are not physically linked to wing-color pattern loci or other genomic regions associated with visual mate preference. Altogether, our results provide evidence for chemosensory divergence between *H. melpomene* and *H. cydno*, two rarely hybridizing butterflies with distinct mate and host plant preferences, a finding that supports a polygenic architecture of species boundaries.

Lepidoptera | smell | taste | butterfly | speciation

How do animals perceive the natural world? While we know that they use multisensory cues to integrate their surroundings and perform basic biological routines, our current understanding of these communication channels is heavily influenced by our own sensory biases. As such, work describing important communication strategies is dominated by descriptions of visual and auditory signals. Comparatively less understood, chemical sensing, which involves both volatile and tactile cues, plays an essential role in a variety of fundamental biological processes (1, 2). For example, chemical communication is important for mate choice (3–5), food choice (6), and host plant choice (7). Moreover, chemosensory communication plays an important role during the early stages of speciation, where it establishes chemical prezygotic barriers that may precede morphological divergence (8, 9). For example, the relatively simple enzymatic mechanisms controlling pheromone synthesis in insects (10) [e.g., *Nasonia* (11, 12)] allow the fast evolution of olfactory signals that drive assortative mating patterns [e.g., *Drosophila* (13); Lepidoptera (14)]. Furthermore, in host-specialized insect systems, chemical perception of host plant signals can serve to further establish ecological species barriers (9). In this way, chemosensory signals may play important roles in both routine behaviors and speciation processes. Despite the fundamental role of chemically mediated behaviors in speciation, few studies have identified chemosensory genes involved in reproductive isolation (9, 15, 16).

To date, most of the work on the genetic basis of chemosensory signaling has been conducted on insects, with an emphasis on *Drosophila* and moths (e.g., *Heliothis* and *Bombyx*). However, in the past few years, the growing accessibility of whole-genome and transcriptome sequencing has allowed us to describe chemosensory genes for a number of new butterfly species. These advances have improved our understanding of the number, diversity, and evolution of chemosensory genes. Interestingly, what has emerged from these studies is a novel view of the chemosensory molecular repertoire of butterflies that reveals an unanticipated, highly complex system, that rivals that found in moths. *Heliconius* butterflies, despite their diurnal and highly visual lifestyle, have more olfaction-related chemosensory genes than moths, whose nocturnal lifestyle requires navigating the landscape while relying largely on chemical cues (17). This apparent paradox likely stems from our limited understanding of

Significance

Insects are dependent on olfactory cues to complete biological processes, such as foraging, oviposition, and mate choice. While extensive experimental evidence supports the importance of chemical cues in these processes, genes involved in chemosensory integration of complex behavioral responses remain largely unknown. Using a combination of differential gene expression and genome-wide signals of gene flow, we describe the chemosensory expression profiles of sensory tissues and identify candidate genes for mate and host plant recognition in a pair of *Heliconius* butterflies. We find that candidate chemosensory genes are physically unlinked from color-pattern genes. Our results suggest the independent evolution of loci associated with the chemosensory and visual systems of *Heliconius*, both potentially mediating behaviors that promote reproductive isolation and downstream speciation.

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how chemosensory genes shape and inform butterfly behaviors, life history, and evolutionary diversity.

Heliconius butterflies, typically recognized for their wing color and pattern mimicry, harbor an impressive diversity of chemosensory molecules, which can be broadly classified into olfactory binding proteins (OBPs), chemosensory proteins (CSPs), olfactory receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs) (Table 1). In *Heliconius*, these molecules likely play important roles in mediating various complex behaviors. For example, *Heliconius* males produce and administer antiaphrodisiacs to females that reduce remating and male harassment (18–20). Additionally, *Heliconius* butterflies feed and oviposit exclusively on *Passiflora* plants for which they display varying degrees of specialization; some are *Passiflora* host plant specialists, and others generalists (21). Furthermore, female butterflies have been documented to evaluate the suitability of host plants prior to ovipositing by "drumming" their forelegs, which contain special sensilla (7), to probe the leaf surface (22, 23). As such, sensory integration of chemical cues emitted by congeners and host plants are essential to the survival of *Heliconius* butterflies.

While current work on *Heliconius* chemosensation has focused on describing the evolutionary diversity of chemosensory genes (CSPs, OBPs, ORs, GRs, and IRs) (7, 35, 36), it has yet to identify individual genes associated with chemically dependent behavioral or biological processes. To better understand the role of chemosensory genes in the establishment of species barriers, we have focused on the recently diverged [~1 Mya (37)], sympatric species pair of *Heliconius melpomene rosina* and *Heliconius cydno chioneus* (Fig. 1) (38–40). These species differ in altitudinal range, host plant ecology, mimicry patterns, microhabitat preference, pheromone chemistry, flight pattern, and wing shape (41). Despite these differences, interspecies mating between *H. melpomene* and *H. cydno* (while rare on a per individual basis) does occur and is generally observed to occur between *H. melpomene* males and *H. cydno* females. These crosses result in sterile females, but fertile F1 males that can back-cross with either species (40, 42, 43). Perhaps because a low rate of hybridization has been ongoing for such a long period, this has resulted in extensive signals of postdivergence gene flow (37, 44, 45). As a result, these two species provide an opportunity to examine the chemosensory correlates of speciation in wild, naturally hybridizing animals.

Our main goal is to identify candidate chemosensory genes underlying reproductive isolation. Previous work has shown that androconial chemicals are important for mate choice, and that there are consistent differences between these species in their androconial wing chemistry across their wide geographic ranges, supporting the hypothesis that chemical signaling is important in reproductive isolation (19, 46). Toward this goal, we first improved the annotation of chemosensory genes using a targeted resequencing approach and later analyzed RNA sequencing (RNA-seq) gene-expression data to identify candidate mate and host plant choice genes. Our experimental design includes three sensory tissue types (antenna, legs, and mouthparts) and three biological groups (males, unmated females, and mated females), representing the recently diverged species pair of *H. melpomene* and *H. cydno* (38–40). With this design we were able to describe species-, sex-, and life-stage– (mated vs. unmated) specific expression profiles that reflect important differences in mate and host plant choice for *H. melpomene* and *H. cydno.*

Combining our gene-expression data with recently published whole-genome sequences for our study species (44), we then examined patterns of differential expression as they relate to genome-wide patterns of admixture. Based on both their expression profiles and associated genomic patterns of speciation, we identified a small number of candidate genes and suggest two strong candidates for species barriers through mate and host plant choice (47, 48). Interestingly, these chemosensory candidates are not in physical proximity to known wing-color pattern genes or other genomic regions associated with visual mate preference. Our work thus suggests that chemosensory traits may have evolved to contribute to reproductive isolation independently from the color-pattern genes and visual discrimination system, providing an additional means for reinforcement in a system characterized by visual mimicry.

Results

Tissue-Specific Expression Recapitulate Sensory Function. We found chemosensory genes belonging to all five families to be expressed in all three tissue types, underscoring the role of these tissues in chemosensation (*SI Appendix*, Table S2). Of the total 252 chemosensory genes, we found more to be expressed in the antennae relative to the legs or mouthparts (45% in antennae, 29.8% in legs, 25% in mouthparts). Nearly 60% were expressed equally

Table 1. Reference table for chemosensory gene categories in *Heliconius*

Categories	Families	Abbreviation	Review
Transporters	Olfactory binding proteins	OBP	Selective in terms of the chemicals they transport, these mainly include volatiles, such as pheromones [e.g., *Drosophila* (24); *Bombyx* (25)].
Small, soluble carrier proteins that move mostly nonsoluble hydrophobic chemicals through the sensillar lymph and to the receptors (26).	Chemosensory proteins	CSP	Less selective than OBPs, they may bind nonvolatile compounds and semiochemicals (27). They also play a role during cuticle development (28) and in immune-related processes (29).
Receptors	Olfactory receptors	OR	Expressed in sensory neurons, these constitute the backbone of the sense of smell. They are responsible for the sensory integration of a wide array of odorant molecules.
Molecules whose function is chemical detection, catalyzing the sensory cascade.	Gustatory receptors	GR	Primarily involved in tasting sweet and bitter compounds (7), they also function as CO_2 receptors (30) and are proposed to be involved in heat avoidance (31).
	Ionotropic receptors	IR	The most primitive class of receptors and, while primarily involved in the smelling and tasting of amines and acids (32, 33), they are also essential for salt detection (34).

For each chemosensory gene category, we provide its abbreviation and a brief review of its role in chemosensation.

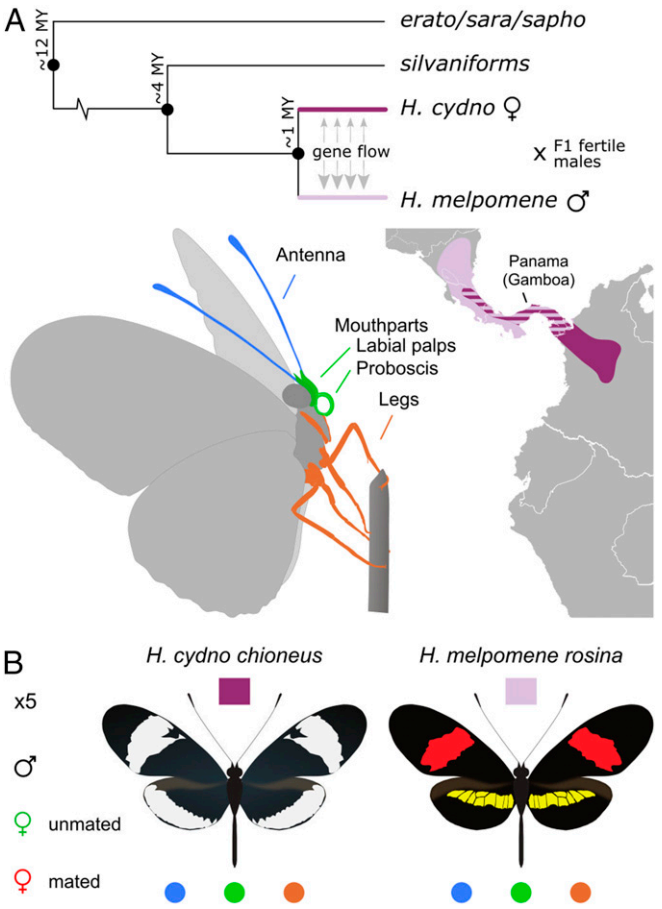

Fig. 1. Phylogenetic relation, geographic distribution, and experimental design to study divergence of gene expression in chemosensory tissues of *H. cydno* and *H. melpomene*. (*A*) *H. cydno and H. melpomene* split ~1 Mya, but male *H. cydno* still occasionally hybridize with female *H. melpomene*, resulting in fertile hybrid males and sterile hybrid females. Genomic patterns of admixture reflect this hybridization, with a generally stronger signal of gene flow from *H. cydno* into *H. melpomene*. We sampled the antennae, legs, and mouthparts (labial palps and proboscis) of *H. cydno* and *H. melpomene* individuals from Panama (Gamboa) in order to study divergence in chemosensory gene expression. (*B*) The experimental design included five replicates for each combination of species, tissues (antennae, legs, and mouthparts) and life stage (males, mated, and unmated females).

in all of the three sensory tissue types, although often at very low levels. The remaining 40% showed variable expression and half of them showed tissue-specific expression (*SI Appendix*, Table S2). ORs and OBPs largely comprised antenna-specific genes, whereas CSPs, GRs, and OBPs generally comprised mouthpart- and leg-specific genes. One of the most prominent features in the antennae was the high expression of ORs, OBPs, and IRs (Fig. 2 and *SI Appendix*, Figs. S2, S3, and S6 and Table S2), a finding that highlights the predominantly olfactory function of antennae. In contrast, the legs and mouthparts displayed very similar chemosensory profiles and were characterized by GR expression (*SI Appendix*, Fig. S4). The latter is in line with results published by Briscoe et al. (7) and underscores the role of legs and mouthparts in gustation. Interestingly, we found *HmGR22*, a *Heliconius*-specific gene and putative bitter receptor, to be highly expressed in all three tissue types, suggesting that this gene might be broadly important for chemosensation. In the mouthparts, we observed that *HmGR56* was overexpressed in both *H. cydno* unmated females and males. It is important to emphasize that while specific expression patterns characterized individual tissue types, we did not observe the exclusive expression of any chemosensory gene family in any of the tissues.

Sex-specific Expression Patterns Suggest OBPs as Mate Recognition Candidates. Approximately 24% of chemosensory genes showed significant expression differences as a function of sex (males vs. unmated females) (Fig. 2 and *SI Appendix*, Tables S3 and S4). We found some of these genes to be differentially expressed between the sexes (regardless of species), suggesting the existence of conserved sex-based chemosensation (Fig. 2). Sex-based expression differences observed between males and females may stem from their different evolutionary needs to recognize odorant volatiles during mate choice and host plant selection. Moreover, genes showing conserved sex-based differences may precede the speciation of *H. melpomene* and *H. cydno*, making them more likely to correspond to broad differences in sex-specific traits. Although we observed a few differentially expressed genes belonging to each chemosensory gene family (Fig. 2 and *SI Appendix*, Tables S3 and S4), OBPs and ORs are the strongest candidates for mate recognition due to their role in detecting volatile compounds, such as pheromones (Table 1). Congruently, the antenna showed the largest number of sex-based expression differences (Fig. 2 and *SI Appendix*, Tables S3 and S4).

Of all of the chemosensory gene families, only OBPs and CSPs showed consistent sex-based differences in expression (Fig. 2). CSPs, however, serve functions outside of chemosensation [e.g., during development (28) and immunity (29)]; this is reflected in their expression across many nonsensory tissues (26), making them less-compelling candidates for mate recognition. Generally, we found OBPs to be expressed in all three tissue types while CSPs were mostly in the mouthparts (Fig. 2). We also observed that genes with consistent sex-based expression patterns were overexpressed in females relative to males, with the mouthparts showing the largest number of differentially expressed genes (Fig. 2). Notably, *HmOBP31* was the only gene to be consistently overexpressed in females of both species in all three tissue types. Three other genes—*HmOBP5, HmOBP52*, and *HmOBP50*—were consistently differentially expressed between the two sexes in at least two tissue types (Fig. 2 and *SI Appendix*, Table S3). Sex-based patterns of gene expression in ORs, GRs, and IRs were observed only within species; none of the genes showed sex-based expression patterns in both *H. melpomene* and *H. cydno* (*SI Appendix*, Tables S3 and S4). Generally, sex-specific differential expression patterns of ORs were restricted to the antennae, except for *HmOR40*, which was overexpressed in the mouthparts of *H. melpomene* males. *HmOR19* and *HmOR5*, two genes previously reported as female-specific ORs in *H. melpomene* legs (7), were found to be overexpressed in *H. cydno* female antennae. Sex-based differential expression patterns of GRs were restricted to the legs and mouthparts. Namely, *HmGR22* was found to be overexpressed in *H. cydno* female legs and mouthparts. The only sex-based difference observed in the IRs was for *HmIR40a*, which was found overexpressed in *H. cydno* males.

Life Stage–Specific Expression Suggests Sensory Shift in Antennae and Mated Females. Approximately 21% of genes showed significant differences as a function of life-stage (unmated vs. mated females) (Fig. 2 and *SI Appendix*, Tables S3 and S4). Differences in chemosensory gene expression between unmated and mated females might reflect a change in sensory priorities from mate choice (in unmated females) to host plant choice for oviposition (in mated females). Like the sex-based expression patterns, we only observed OBPs and CSPs to be consistently differentially expressed between life-stages for both *H. melpomene* and *H. cydno* (Fig. 2 and *SI Appendix*, Tables S3 and S4). Four OBPs (*HmOBP5*, *HmOBP31*, *HmOBP42*, and *HmOBP47*) were consistently differentially expressed between unmated and mated

female antennae across species. *HmOBP5*, *HmOBP31*, and *HmOBP42* were overexpressed in unmated females, while *HmOBP47* was overexpressed in mated females (Fig. 2 and *SI Appendix*, Table S3). *HmOBP31* is particularly interesting because it is overexpressed in all three tissues for unmated females (Fig. 2). Moreover, *HmOBP31* and *HmOBP42* are strong candidates for female mate choice because they are overexpressed in unmated female antennae relative to both mated females and males. Several genes showed similar expression patterns in both the sex-specific and life-stage analyses (Fig. 2 and *SI Appendix*, Tables S3 and S4). The latter is due to similarities between the expression patterns of mated females and males; this causes life-stage patterns of gene expression to greatly overlap with the sex-specific patterns.

Similar to what we observed in the sex-specific analysis, life-stage—based patterns of gene expression in ORs, GRs, and IRs were observed only within species; none of the genes showed life-stage–based expression patterns across species (*SI Appendix*, Tables S3 and S4). Most life-stage expression differences in ORs were restricted to the antenna, except for *HmOR37*, which was overexpressed in the legs of *H. melpomene*-mated females. All life-stage differences for GRs show a higher expression in

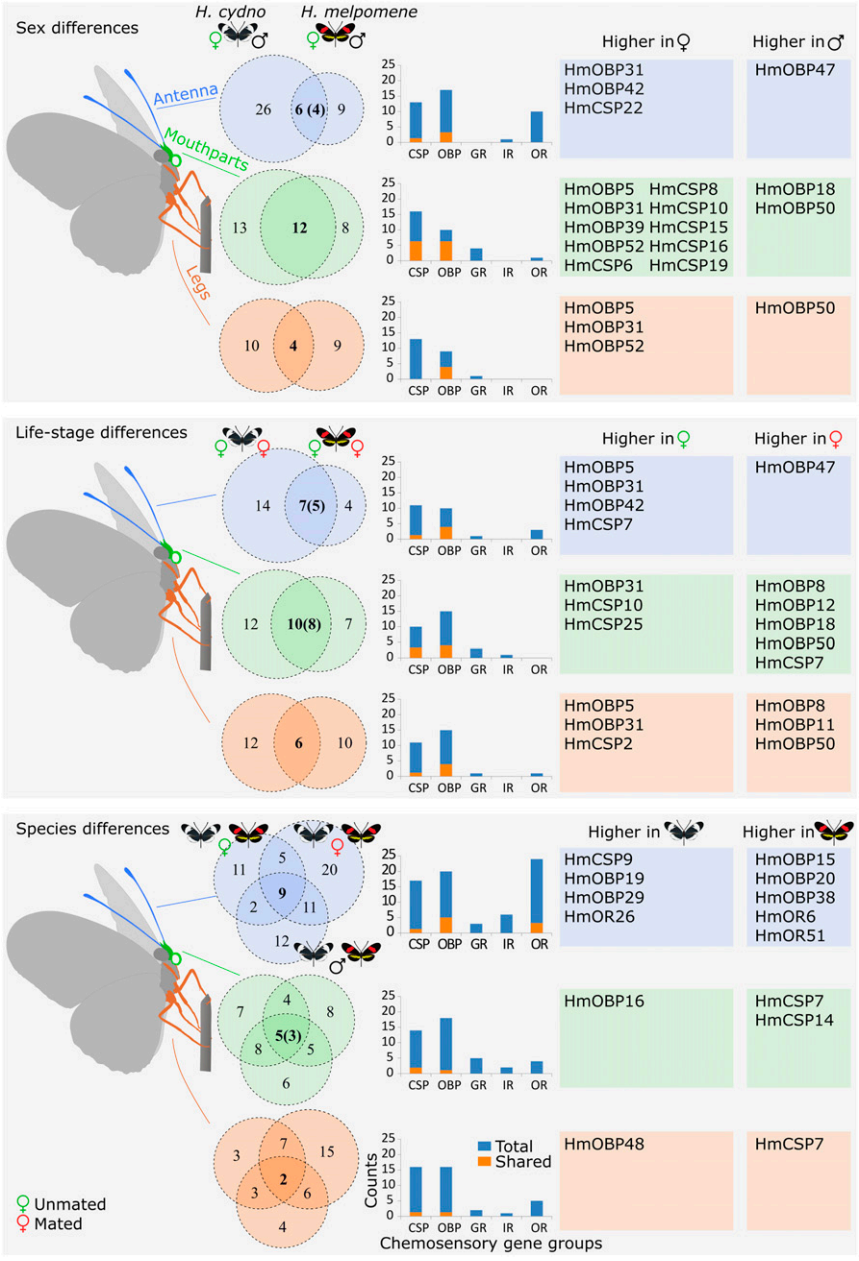

Fig. 2. Overview of differentially expressed genes by tissue type, sex, life-stage, and species in *H. cydno* and *H. melpomene*. Venn diagrams show the number of genes differentially expressed and shared among comparisons (numbers between parentheses match the genes that are consistently differentially expressed in the same direction across sex, life-stage, or species). Bar plots indicate differentially expressed genes in each chemosensory gene family. The blue bars indicate the total gene count and the nested orange bars correspond to the genes in the overlap region of each respective Venn diagram. Tables on the right list the names of genes that are consistently differentially expressed in the same direction across sex, life-stage, or species; these match the overlap region of each respective Venn diagram.

unmated females relative to mated females. The only life-stage based difference observed in the IRs was for *HmIR25a*, which was overexpressed in the mouthparts of *H. cydno* unmated females. More generally, we again observed significant overlap between sex-specific and life-stage–specific patterns.

Species-Specific Expression Patterns Identify Potential Species Barriers. Approximately 40% of genes show species-specific differential expression patterns for at least one biological group (males, unmated females or mated females) (Fig. 2 and *SI Appendix*, Tables S3 and S4). A subset of these genes showed expression patterns strictly as a function of species (*H. melpomene* vs. *H. cydno*); these were conserved across all three biological groups (males, unmated females, and mated females). Most of these species-specific differences were observed in the antenna, particularly in ORs and OBPs. More specifically, in the antennae, *HmOBP15*, *HmOBP20*, *HmOBP38*, *HmOR6*, and *HmOR51* were overexpressed in *H. melpomene* and *HmCSP9, HmOBP19*, *HmOBP29*, and *HmOR26* were overexpressed in *H. cydno*. In the legs, we found *HmCSP7* to be overexpressed in *H. melpomene* and *HmOBP48* to be overexpressed in *H. cydno.* In the mouthparts, *HmCSP7* and *HmCSP14* were overexpressed *H. melpomene* and *HmOBP16* was overexpressed in *H. cydno.*

More broadly, the number of overexpressed transporters (OBPs and CSPs) and receptors (ORs, GRs, IRs) in the antenna of *H. cydno* ($n = 60$) was greater than that observed in *H. melpomene* ($n = 46$). This difference was mainly driven by the male and mated female categories. We found 13 overexpressed transporters in *H. cydno* males compared to 5 in *H. melpomene* males (*SI Appendix*, Table S3), and 15 overexpressed receptors in *H. cydno* mated females compared to 5 in *H. melpomene* (*SI Appendix*, Table S4). It is possible that this increased chemosensory expression corresponds to both host plant generalist behavior and strict conspecific mate choice preferences in *H. cydno* (21, 40, 42, 43), behaviors that might require increasingly specialized and sensitive chemosensory discrimination genes.

Patterns of Differential Expression in the Context of Admixture. Of the 252 chemosensory genes in our dataset, we were able to confidently assign genomic admixture levels (f_d) to 212 (44) (Fig. 3*A*). From these 212 genes, we extracted 149 genes that were significantly differentially expressed between *H. melpomene* and *H. cydno* in any of the three tissues (antennae, legs, and mouthparts) and for any of the three biological groups (males, mated females, and unmated females) (Fig. 2, species differences). These 149 genes were sorted into "genes overexpressed in *H. melpomene*" and "genes overexpressed in *H. cydno*." Of these 149 chemosensory genes, 8.72% ($n = 13$) were both significantly overexpressed and had low admixture ($f_d \leq 0$) (Fig. 4). This subset was composed of one OBP, three GRs, and four ORs. Only one chemosensory gene, however, showed species-level expression patterns consistent across biological groups and tissue type: *HmOBP20* (q-value ≤ 0.001 for all comparisons). *HmOBP20* was found to be overexpressed in *H. melpomene* antennae relative to *H. cydno,* and this pattern was observed in males, mated females, and unmated females (Fig. 5). As such, *HmOBP20* presents a compelling candidate for species-specific recognition in *H. melpomene*.

Three GRs were significantly differentially expressed with low admixture ($f_d \leq 0$) (*HmGR63*, *HmGR64*, and *HmGR56*) (Fig. 6*A*). Here, the most pronounced difference in expression is observed for *HmGR56* in *H. cydno* male mouthparts. Generally, *HmGR56* presents a trend toward increased expression in *H. cydno* legs relative to *H. melpomene* (Fig. 6*B*). The latter is noteworthy because *HmGR56* is a *Heliconius*-specific GR putatively involved in synephrine (a common plant alkaloid) recognition (7) and may present an interesting candidate for host plant recognition. Four ORs were both significantly overexpressed and had low admixture ($f_d \leq 0$) (*HmOr16*, *HmOr25*, *HmOr32*, and *HmOr43*) (Fig. 7). Here, the most pronounced differences in expression are observed for ORs expressed in the antennae (*HmOr16*, *HmOr32*, and *HmOr43*).

Genomic Locations of Chemosensory Genes and Linkage Disequilibrium. Generally, we found that chemosensory genes occur as small clusters, were present on every chromosome, and were spread across the genome (Fig. 3*A*). These clustering patterns were in line with expectations for genes originating from duplications as per the birth–death model of gene family evolution (49). On a finer scale, some clear differences between gene families emerge. CSPs, for example, showed a strong clustering pattern to specific areas of the genome. This contrasts with GRs, which were represented by many small clusters of genes across the genome. Similarly, OBPs appeared in small clusters, while ORs and IRs form no clusters but were spread out across the genome. Generally, genes clustering together were more closely related. One gene (*HmOR3*) mapped to the sex chromosome (Fig. 3*A*). *HmOR3* is overexpressed in the in antenna of *H. cydno*-mated females relative to *H. melpomene*-mated females (*SI Appendix*, Table S4).

Genetic associations between preference and mating cue loci may promote assortative mating in diverging species (9, 43, 50, 51). In *Heliconius*, for example, the major color-pattern gene *optix* is associated with visual assortative mating behavior (51). As such, we identified chemosensory genes within 1 Mb of key wing-pattern formation genes (*WntA*, *optix*, and *cortex*); these genes are also involved in divergent natural selection (52, 53). Near *WntA*, a gene involved in variation in forewing band shape (52), we identified three chemosensory genes: *HmOR23* (89 kb), *HmGR62* (772 kb), and *HmOR49* (952 kb). Near *optix*, a transcription factor controlling ommochrome development (53), we found one gene: *HmOBP40* (632 kb). Near *cortex*, a gene involved in determining the presence of a yellow hindwing bar and white forewing (54), two chemosensory genes were identified: *HmOR55* (498 kb) and *HmOR12* (546 kb). While close proximity (within 1 Mb) could indicate that the chemosensory genes are physically linked to the wing-pattern genes (in linkage disequilibrium, LD), none of these chemosensory genes were identified as candidates based on expression and admixture data.

Due to the association of color pattern with mate-preference behavior in *H. melpomene* and *H. cydno* (51), we specifically explored the levels of LD between our chemosensory genes ($n = 252$) and the *optix* color pattern interval (Fig. 3*B* and *SI Appendix*, Fig. S7 and Table S5). Additionally, we tested LD levels between a group of randomly selected, nonchemosensory genes ($n = 300$) and the *optix* color pattern interval. Compared to the randomly selected group of 300 genes, the 252 chemosensory genes did not show an increase in LD among each other or with the divergently selected *optix* locus. The average r^2 among the chemosensory genes was 0.19 ± 0.07 and closely matches the r^2 observed among the randomly selected genes (0.19 ± 0.09). The average r^2 of the chemosensory and random genes with the divergent *cis*-regulatory region of *optix* was 0.27 ± 0.11 and 0.29 ± 0.13, respectively. Of the chemosensory loci identified using differential expression and admixture, we found that *HmGR56* ($r^2 = 0.45$) and *HmOr32* ($r^2 = 0.46$) had the strongest association with *optix* and were in the top 12% highest LD with *optix*. *HmOBP20* also showed increased LD with the *optix* region ($r^2 = 0.35$) and was in the top 26% highest LD with *optix*. Among the chemosensory genes, the strongest association, however, was observed for *HmIR75d* ($r^2 = 0.64$), a gene that has no salient expression pattern (Fig. 3*B* and *SI Appendix*, Fig. S7 and Table S5).

Discussion

Despite the essential role of chemosensory communication in insect behavior, we still know relatively little about the genetics

underlying chemosensory discrimination of mate and host plant choice and their potential involvement in speciation. *Heliconius* butterflies exhibit chemically mediated behaviors (e.g., mate choice, mate searching, and host plant choice for oviposition) that are vital for survival and reproduction. Recently diverged species within this genus provide an excellent opportunity to study the role of chemosensation in speciation. Here, we have investigated chemosensory divergence in two recently diverged *Heliconius* butterfly species (*H. melpomene* and *H. cydno*) that show both strong assortative mating and use different larval host plants. By combining gene expression and genetic divergence data in this species pair, we have identified strong candidate genes for mate and host plant choice that may have important implications for the establishment of chemically mediated species barriers.

A strength of this study is the use of both RNA expression and DNA admixture data to identify chemosensory genes correlated with the early stages of speciation. Species-based expression differences may stem from a variety of life-history variables: For example, differences in adaptations for mate choice, as well as host plant preferences. These differences are particularly important for butterflies on the verge of speciation, where chemically mediated signals may be essential for the establishment of species barriers. Multiple studies support the importance of chemically mediated species boundaries (9), which indicate that species can differ in both the type and relative abundance of chemosensory genes that facilitate the detection of species-specific chemical cues, generally volatile odorants. In agreement with this, most species-specific differences in expression were observed in the antennae and were characterized by OBPs and ORs, as would be expected if these genes were involved in the recognition of volatiles (e.g., pheromones).

Despite being closely related, *H. cydno* and *H. melpomene* display key differences in reproductive behavior. They show strong premating isolation and, in laboratory crosses, the two species hybridize asymmetrically, with hybrids more often resulting from *H. melpomene* males and *H. cydno* females (40, 42, 43). Second, *H. cydno* is a host plant generalist and, in Central America, *H. melpomene* is a host plant specialist (21). These differences in mating and oviposition may cause broad patterns of differential expression between *H. cydno* and *H. melpomene*. In particular, we observed three times more genes overexpressed in the antenna of *H. cydno* males and mated females relative to the same categories in *H. melpomene*. This increased chemosensory expression in *H. cydno* might, for example, confer males and females of this species a greater ability to distinguish conspecific mates or recognize a wider range of host plants. Future work on populations of *H. melpomene* from eastern parts of the range (which are more host plant generalist) might provide a means to test whether expansions in gene expression are associated with host plant use.

In order to select the strongest candidates for mate and host plant choice, we identified genes that showed both strong differential expression and were located in regions of the genome with low admixture ($f_d \leq 0$). We hypothesized that chemosensory genes found in this cross-section of our data were likely to be involved in species-specific processes. Using this strategy, we identified eight candidate genes for species-specific processes: OBPs ($n = 1$), ORs ($n = 4$), and GRs ($n = 3$). OBPs and ORs are known to be involved in pheromone detection and GRs are likely under strong divergent selection due to specialized host-plant usage. This contrasts with CSPs and IRs, which are understood to have a more general role in chemosensation (Table 1). *HmOBP20*, however, stands out for being the only gene showing both significant differential expression at the species-level and low admixture ($f_d \leq 0$). *HmOBP20* is overexpressed in *H. melpomene* antennae relative to *H. cydno* and this pattern is consistently found across biological groups (Fig. 5). The observed antennae-specific expression of *HmOBP20* is in line with the hypothesized role of this gene in volatile pheromone detection. Work on the silkmoth (*Bombyx mori*) homolog *BmOBP20* underscores the importance of this gene in chemosensation (55). More recently, work on the *Drosophila* homolog (*OBP69a*) suggests

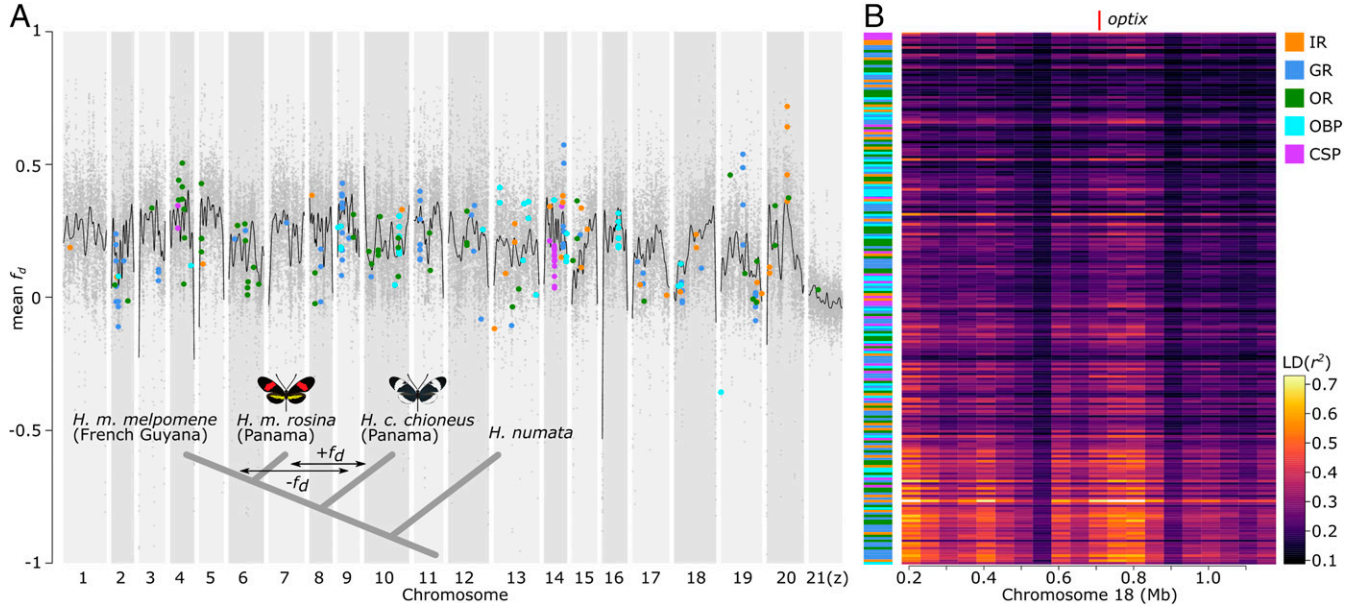

Fig. 3. Genomic patterns of divergence between *H. melpomene* and *H. cydno*. (*A*) Per chromosome genomic patterns of admixture between *H. cydno* and *H. melpomene* (bottom left corner; population and relationships used in the admixture analysis). For all 21 chromosomes in the *Heliconius* genome (*x* axis), we show genome-wide admixture values (*y* axis; mean f_d) calculated in 20-kb windows and per chromosome cubic splines (black lines). Chemosensory gene families are color-coded as indicated in the legend. (*B*) LD (r^2) map of chemosensory genes and the color-pattern gene *optix*. On the *y* axis chemosensory gene families are color-coded as per the legend and ordered according to their f_d value (highest f_d at bottom). The *x* axis indicates a 1.2-Mb window around *optix* (red line) analyzed in 50-kb windows. For chemosensory gene names associated with *B*, see *SI Appendix*, Fig. S7 and Table S5.

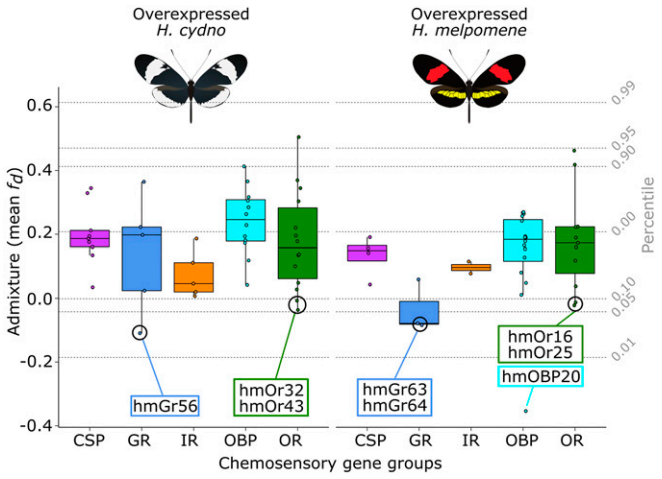

Fig. 4. Admixture of chemosensory genes overexpressed in *H. cydno* and *H. melpomene*. Only chemosensory genes significantly overexpressed for each species are plotted. Admixture (*y* axis) is measured using a mean f_d value was calculated from a 50-kb window around the transcription start site of each gene. Genes are considered to not show signals of admixture when $f_d \geq 0$; chemosensory genes with $f_d \leq 0$ are identified by their name. Mean genome f_d and percentiles are indicated using dashed lines.

that the expression of *OBP69a* is regulated by *cis*-vaccenyl acetate (a male-specific *Drosophila* pheromone) and experimentally demonstrates that expression levels of *OBP69a* modulate male-male aggression and female receptivity (56). Moreover, *HmOBP20* was previously identified to be in a region of high divergence between *Heliconius elevatus* and *Heliconius pardalinus* (57), two other recently diverged species in the genus. As such, *HmOBP20* is a strong candidate for species-specific recognition of pheromones in *Heliconius*. The molecular mechanisms controlling divergence patterns at this gene, however, are likely very complex. Sequence-level comparisons of *HmOBP20* across *Heliconius* species indicate that the protein structure is highly conserved (no amino acid changes), suggesting that patterns of divergence at *HmOBP20* are driven by gene regulatory elements.

Due to the highly specialized relationship between *Heliconius* and *Passiflora*, chemosensory genes involved in plant volatile and nonvolatile recognition are likely under strong divergent selection and may represent important species barriers. This is particularly true for females, who select ovipositing sites in part by tasting the putative host plant surface with their forelegs, a behavior known as "drumming" (22, 23). In agreement with these behavioral observations, we find that in *H. cydno* (a plant generalist) *HmGR22*, *HmGR3*, *HmGR56*, and *HmGR5* are overexpressed in the legs and mouthparts of females. It is noteworthy that no GRs were found to be overexpressed in the males, suggesting that overexpression of GRs may be exclusive to specialized female sensilla. Of these four GRs, *HmGr56* emerges as a candidate for host plant discrimination in *H. cydno*. *HmGr56* was found to have low admixture ($f_d \leq 0$) and to be overexpressed in *H. cydno* male mouthparts and mated/unmated female legs (Fig. 6*A*). Generally, however, *HmGr56* expression was higher in *H. cydno* legs relative to *H. melpomene* (Fig. 6*B*). Moreover, the expression of *HmGr56* was restricted to the legs and mouthparts, as would be expected if the gene were involved in recognizing contact cues associated with plant identity. Briscoe et al. (7) had previously identified *HmGr56* to be highly expressed in female legs and described the gene as part of a cluster of putative paralogues of a *Papilio xuthus* GR known to be involved in synephrine recognition, a common plant alkaloid (58). Taken together, these data suggest that *HmGr56* may be involved in host plant discrimination.

As a result of the sophisticated visual system (59) and wing-pattern diversity in *Heliconius* butterflies (60), speciation work in the *Heliconius* has focused on the three genes thought to control most wing coloration (*optix* [chromosome 18, *chr18*], *WntA* [*chr10*], and *cortex* [*chr15*]). In terms of mating behavior, a recent study identified three large-effect quantative trait loci [QTLs] associated with largely visual-based male mating preference, including one in close physical proximity to *optix*, the gene responsible for differences in red pattern variation between *H. cydno* and *H. melpomene* (51). While this and other studies (40, 43) support a strong role for visual cues in *HeIiconius* butterfly behavior, the role of chemosensory communication in establishing and maintaining species barriers remains understudied. We propose that *HmOBP20* represents an independently evolved strategy for discrimination and reinforcement of species barriers in *Heliconius*. In addition to presenting strong species-specific genomic signals, *HmOBP20* is not physically linked with any of the classic color pattern genes (*optix* [*chr18*], *WntA* [*chr10*], and *cortex* [*chr15*]), nor is it linked to any of the previously identified male preference QTLs (*chr1*, *chr17*, *chr18*). Instead, it is located on a different chromosome (*chr19*). This finding supports a multimodal system of speciation that involves both chemical and visual cues, a conclusion that is emerging from studies looking at chemical differences in wing pheromones and the role that they play in reproductive isolation (46, 61–63).

This work provides a foundation for elucidating the mechanistic basis of chemosensation in Lepidoptera, as well as understanding how chemosensory communication shapes insect behaviors and establishes species barriers. The admixture and expression-level genomic signatures observed at these candidate genes suggest that key changes underlying chemically mediated adaptations are likely found in the regulatory architecture controlling the time, level, and place of expression of these chemosensory genes. This mechanism is similar to what has been recently reported for wing-color pattern variation in *Heliconius* (64, 65). Our chemosensory candidates are not found in physical proximity to color pattern genes or genomic regions associated with visual mate preference but show increased LD with the *optix* interval. We thus suggest their likely independent evolution from the visual reinforcement system and propose that these chemosensory genes work in tandem with color pattern (62, 66) and vision genes (59, 67) to mediate reproductive isolation in

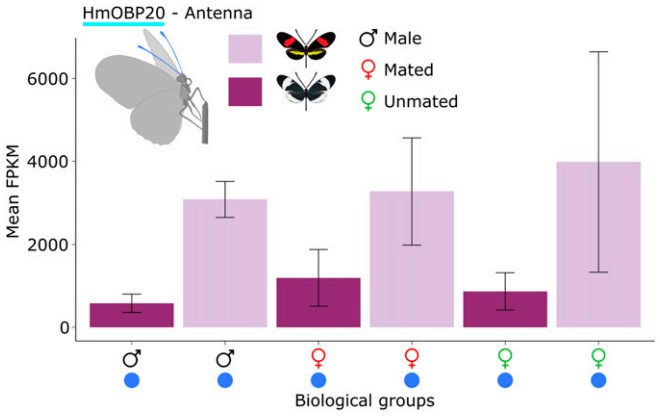

Fig. 5. Expression levels for the odorant binding protein HmOBP20. *HmOBP20* shows increased expression in the antennae for all three biological groups (males and mated and unmated females) of *H. melpomene* relative to *H. cydno*. Expression of *HmOBP20* is significant (and almost exclusive) to the antennae and very low (or not present) in the legs and mouthparts. Error bars in both figures were calculated using SD values.

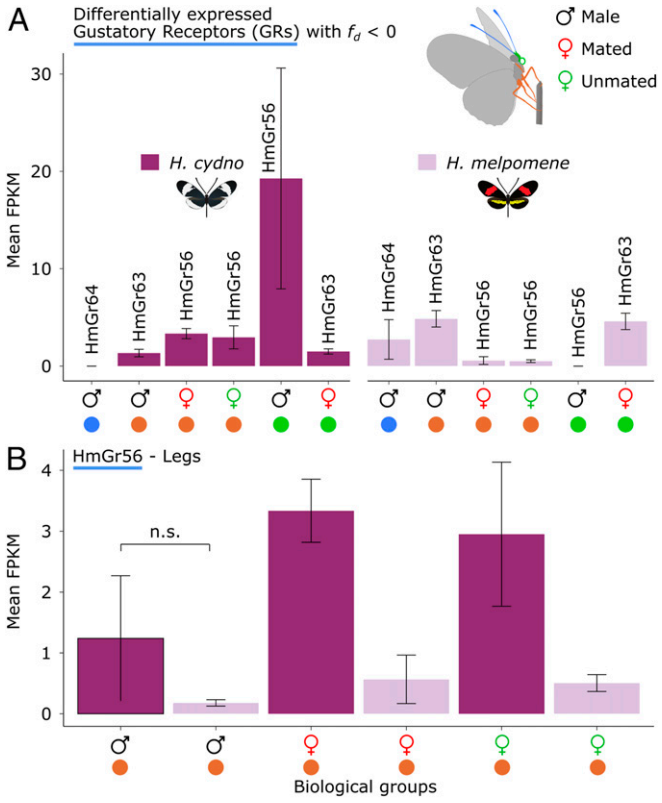

Fig. 6. Expression levels for GRs of interest. (*A*) Expression levels of differentially expressed GRs with $f_d \leq 0$ between *H. cydno* and *H. melpomene*. (*B*) Expression levels of *HmGR56* in the legs. Note that *HmGR56* was not found to be significantly differentially expressed in male legs but displays the same trend. Error bars were calculated using SE values.

Heliconius. While our data indicate that OBPs, ORs, and GRs are the most compelling candidates for mate and host plant choice, these candidates have yet to be validated. Future efforts to elucidate the molecular basis of chemosensory perception will focus on the role of individual genes in these behaviors.

Materials and Methods

Sampling, RNA Extraction, and Sequencing. We bred 15 *H. melpomene* and 15 *H. cydno* butterflies in seminatural conditions from November 2013 to March 2014 in Gamboa, Panama. Unmated females were 2-d-old at sampling and both males and mated females were 5-d-old; females were mated within the first 2 d after eclosing. For each individual we collected tissues of three sensory tissue types: Antenna, legs (all six legs), and mouthparts (including the proboscis and labial palps). We extracted RNA separately from each of the three sensory tissue types for a total of 90 individual RNA extractions representing 3 biological groups (5 males, 5 unmated females, and 5 mated females) of 2 closely related species (15 *H. melpomene* and 15 *H. cydno*) (*SI Appendix*, Fig. S1). RNA was extracted using a TRIzol RNA isolation protocol followed by additional purification using a RNeasy minikit (Qiagen). Two Illumina libraries, each representing 45 individually barcoded RNA extractions for either *H. melpomene* or *H. cydno*, were generated using the Illumina TruSeq RNA sample preparation kit. Each library was sequenced three times on a HiSeq2500 Illumina system for a total of six Illumina sequencing lanes (50 bp single read) (*SI Appendix, Detailed Materials and Methods*).

Targeted Resequencing and Chemosensory Gene-Annotation Improvement. We improved the chemosensory gene models by producing a target RNA resequencing dataset for the genes of interest (*SI Appendix*, Fig. S1). For a subset of the sampled *H. melpomene* butterflies (two males, two unmated females, and two mated females), we pooled equal amounts of RNA extracted from the antenna, legs, and mouthparts for each individual. The pooled samples (n = 6) were sent to Roche for targeted enrichment of sequencing libraries using the NimbleGen SeqCap protocol (68) followed by sequencing on the Illumina Hiseq2500 platform (100 bp paired end). We then used the targeted capture data to improve the annotation of the chemosensory genes, in particular the beginnings and ends of genes, and to find previously unannotated genes in the Hmel2 genome (*SI Appendix*, Table S1). With this improved chemosensory gene data set, we were able to accurately annotate genes and analyze our RNA-seq data (*SI Appendix, Detailed Materials and Methods*).

Raw Data Processing and Differential Expression Analyses. The Illumina RNA-seq data were aligned to the Hmel2 genome using "TopHat" (q-value threshold = 0.05; for per gene q-values, see Dataset S1) and differential expression analyses were performed with Cufflinks (69). Differential expression analyses were conducted by tissue and biological group as follows. For the tissue-specific expression analyses, we combined data for all 30 butterflies and compared across tissue types. For species-, sex-, and life-stage–specific expression analyses, the data were analyzed for each tissue type independently (antennae, legs, or mouthparts). Species-specific differences were assessed by comparing males, unmated females, or mated females of *H. melpomene* to their biological equivalents in *H. cydno*. Here, differences in chemosensory gene expression between closely related species were hypothesized to reflect differences in mate or host plant choice. Sex-specific differences were assessed by comparing males and unmated females. This comparison was established to identify genes potentially central to both female- and male-choice. Life-stage–specific differences were assessed by comparing unmated and mated females. Here, differences between unmated and mated females were hypothesized to reflect a shift in sensory focus from mate searching (unmated females) to host plant detection for oviposition (mated female). Expression plots based on FPKM (fragments per kilobase of transcript per million mapped reads) values were created using the "heatmap.2" function of the "gplots" package in R (70) (*SI Appendix, Detailed Materials and Methods*).

Admixture and LD Analyses. In order to further narrow down genes underlying species-level recognition, we compared results from our species-level differential expression analyses to admixture estimates for the sympatric *H. melpomene–H. cydno* pair from Panama (44). The admixture values (f_d) are based on the ABBA-BABA test (71), which measures an excess of derived allele sharing between the sympatric nonsister taxa compared to an allopatric population of *H. melpomene* from French Guiana. We included *H. numata* as an outgroup (Fig. 3*A*). Negative f_d values indicate low admixture or sharing of derived alleles between the sympatric Panama populations of *H. melpomene* and *H. cydno*, whereas positive f_d values indicate high admixture. The f_d values were calculated for 100-kb windows with a step size of 20 kb. For chemosensory genes, overlapping windows were averaged. With this approach, we were able to identify chemosensory genes that were both significantly differentially expressed (overexpressed) and presented low admixture between the two species. Chemosensory genes at the intercept of these two estimates are likely to be involved in species-specific processes. Finally, because the color-pattern locus *optix* has been shown to be associated with mate preference behavior in *H. melpomene* and *H. cydno* (51), and linkage between chemosensory and color-pattern genes could facilitate the forming of smell-based prezygotic barriers (43), we tested for the nonrandom association of chemosensory loci (50 kb around the transcription

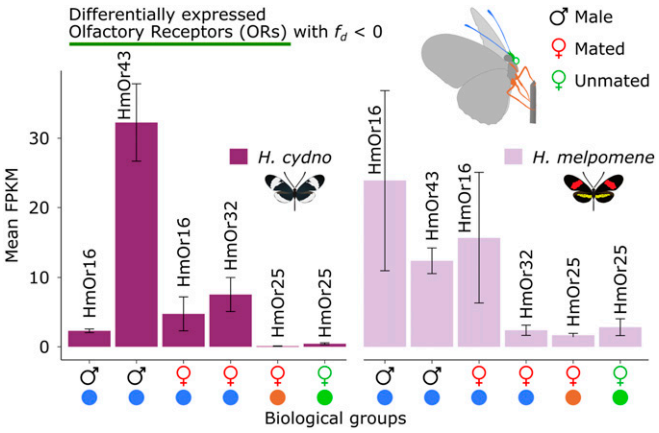

Fig. 7. Expression levels for ORs of interest. Expression levels of differentially expressed ORs with $f_d \leq 0$. Error bars were calculated using SE values.

start site) at and around the *optix* gene (1,000-kb region) in 50-kb windows. Pairwise comparisons of SNPs within each 50-kb window were averaged to produce a mean r^2 (*SI Appendix, Detailed Materials and Methods*). Finally, we evaluated the strength of LD associations for our chemosensory genes by calculating background r^2 levels using 300 randomly sampled, non-chemosensory genes across the genome. We then compared r^2 levels for our chemosensory genes against this null r^2 distribution.

Data Availability. The data that support the findings of this study are openly available on the National Center for Biotechnology Information Sequence Read Archive repository under the BioProject accession numbers PRJNA577441 (RNA-seq data) (47) and PRJNA577716 (targeted resequencing data) (48). Editable excel and text files associated with the data here presented are available on the Open Science Framework database (DOI: 10.17605/OSF.IO/2MB38) (72).

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