Evolution of Sex-Biased Gene Expression and Dosage Compensation in the Eye and Brain of *Heliconius* Butterflies

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Abstract

Differences in behavior and life history traits between females and males are the basis of divergent selective pressures between sexes. It has been suggested that a way for the two sexes to deal with different life history requirements is through sex-biased gene expression. In this study, we performed a comparative sex-biased gene expression analysis of the combined eye and brain transcriptome from five *Heliconius* species, *H. charithonia*, *H. sara*, *H. erato*, *H. melpomene* and *H. doris*, representing five of the main clades from the *Heliconius* phylogeny. We found that the degree of sexual dimorphism in gene expression is not conserved across *Heliconius*. Most of the sex-biased genes identified in each species are not sex-biased in any other, suggesting that sexual selection might have driven sexually dimorphic gene expression. Only three genes shared sex-biased expression across multiple species: ultraviolet opsin *UVRh1* and orthologs of *Drosophila Krüppel-homolog 1* and *CG9492*. We also observed that in some species female-biased genes have higher evolutionary rates, but in others, male-biased genes show the fastest rates when compared with unbiased genes, suggesting that selective forces driving sex-biased gene evolution in *Heliconius* act in a sex- and species-specific manner. Furthermore, we found dosage compensation in all the *Heliconius* tested, providing additional evidence for the conservation of dosage compensation across Lepidoptera. Finally, sex-biased genes are significantly enriched on the Z, a pattern that could be a result of sexually antagonistic selection.

Key words: **gene expression evolution, evolutionary rates, Z-chromosome enrichment, vision, UV opsin.**

Article

Introduction

Differences between females and males, denoted as sexual dimorphism, are present throughout the animal kingdom. This dimorphism can manifest itself in a variety of ways, including differences in shape and coloration (Williams and Carroll 2009), physiological and behavioral responses, or differences in sensory perception. Sexual dimorphism can arise because of differences in sex-specific natural selection or sexual selective pressures, leading to different fitness optima between the sexes, a phenomenon that has been described as sexual antagonism (Rice 1984; Charlesworth et al. 1987). The conflict arising from different fitness optima between sexes and the fact that females and males have an almost identical genome, could be resolved through sex-specific gene expression. A gene is classified as being sex-biased when its expression is unique to one sex or if it is significantly differentially expressed between the sexes (Ellegren and Parsch 2007).

Sex-biased gene expression can have a major influence on sexually dimorphic morphological traits. For example, during *Drosophila biarmipes* development, sex-biased expression of *yellow* and *ebony* in the forewing causes the presence of male-specific wing spots, which are used to catch the female's attention during courtship behavior (Gompel et al. 2005). Sex-specific behavior can also be achieved through sex-biased gene expression. For example, in the silk moth *Bombyx mori*, a number of odorant receptors expressed in the antennae show sex-biased expression. The odorant receptor BmOR19 shows a very strong female-biased expression in the antennae (Wanner et al. 2007; Jordan et al. 2009). The odor ligand activating BmOR19 is linalool, a ubiquitous plant, and flower volatile. Electro-antennogram recordings revealed that female antennae react more strongly to linalool than male antennae, suggesting that linalool is an important cue for host seeking in females (Bisch-Knaden et al. 2014).

In addition to sexual dimorphism in the olfactory system, there are several other forms of sexual dimorphism known in Lepidoptera (butterflies and moths), including in wing patterns, wing coloration and size, predator palatability, acoustic perception, and eye size (Rutowski 2000; Kemp 2008; Rodríguez-Loeches et al. 2009; Allen et al. 2011; Everett et al. 2012; Nadeau 2016). In Neotropical butterflies of the genus *Heliconius*, the opsin detecting ultraviolet (UV) light went through a duplication event, resulting in two opsin genes, *UVRh1* and *UVRh2* (Briscoe et al. 2010). Through eye electrophysiology it was found that in *H. erato* the two UV rhodopsins, consisting of an opsin and an 11-*cis*-3-hydroxyretinal chromophore, have different wavelengths of peak absorption. UV1 rhodopsin absorption peaks at 355 nm and UV2 rhodopsin at 390 nm. Electrophysiological recordings

and immunohistochemistry staining revealed that while UV1 and UV2 are female-specific, UV1 is completely absent in males (McCulloch et al. 2016). In *H. sara* and *H. charithonia*, like in *H. erato*, male expression of UV1 is also absent. *Heliconius doris* females and males express the two UV opsins, whereas in *H. melpomene* only UV1 is expressed in both sexes (McCulloch et al. 2017). The expression diversity observed in *Heliconius* UV opsins motivated the first objective of this study: to uncover the full extent of transcriptome sexual dimorphism in eye and brain tissue.

Our second objective was to investigate the presence of dosage compensation in the *Heliconius* clade. In organisms having sexes with a heterogametic chromosome system, either XX/XY or ZZ/ZW, sex-linked genes in the heterogametic sex have close to half the expression of autosomes or the homogametic sex in the absence of gene expression compensation. Since the sex chromosomes are thought to have evolved from the autosomes, it is hypothesized that before the degeneration of the W or the Y, gene interactions between the autosomes and the Z were free from a dosage effect. Therefore, to avoid disruption of autosome-to-sex chromosome interactions after the formation of the W or Y, and to equalize the Z or X between sexes, a dosage compensation mechanism is expected to evolve (Ohno 1967).

The evolution of dosage compensation is thought to be tightly linked to the mating system type and the strength of sex-specific selection. These two factors directly influence the effective population size of the sex chromosomes and thus the dynamics between drift and selection, which in turn can determine the emergence of a dosage compensation mechanism (Wright and Mank 2013). For example, when sexual selection is stronger in males, which is usually triggered by strong male competition for female accessibility, the reproductive success in males will be higher than in females. Under such circumstances, when the male is the homogametic sex, like in ZZ/ZW systems, the effective population size (Ne) of the Z will be much smaller in comparison to systems where males are the heterogametic sex, like in XY chromosomal systems. Since larger Ne results in faster evolution, the larger population size of the X in comparison to the Z would facilitate mutations leading to faster evolution of dosage compensation in XX/XY than in ZZ/ZW systems (Mullon et al. 2015).

In some ZZ/ZW systems like in birds the lack of dosage compensation in the Z of the heterogametic sex is well established (Itoh et al. 2007). On the other hand, in lepidopterans (butterflies and moths) the presence of dosage compensated sex chromosomes has been reported, with the Z-chromosome in the heterogametic sex having comparable expression to the homogametic sex (Smith et al. 2014; Walters et al. 2015; Gu et al. 2017; Huylmans et al. 2017). Variability of dosage compensation has been observed depending on the organ examined, the diversity of mating systems found in a clade, and the degree of sexual selection experienced by each sex at a particular developmental stage (Itoh et al. 2007; Mullon et al. 2015).

To begin to understand how sexual dimorphism in the visual system is evolving and how conserved dosage compensation in somatic tissue of *Heliconius* butterflies is, we sequenced combined eye and brain tissue transcriptomes from five *Heliconius* species: *H. charithonia*, *H. sara*, *H. erato*, *H. doris*, and *H. melpomene*. These five species represent five of the seven main clades identified in the *Heliconius* phylogeny with divergence times ranging from 5.5 to 11.8 Ma (Kozak et al. 2015) (fig. 1).

We find that the degree of sex-biased expression in *Heliconius* eye and brain is not conserved across the species studied and that the overlap of the sex-biased genes identified across species is significantly lower than expected transcriptome-wide. Additionally, rates of evolution are significantly higher in sex-biased compared with unbiased genes, with a number of these sex-biased genes showing evidence of positive selection for gene expression divergence. Our data suggest that female-biased genes are driving the rapid evolutionary rates observed in sex-biased genes in some *Heliconius* species, whereas male-biased genes are driving them in others. These results suggest that sexual dimorphism in gene expression is influenced by an organism's sex-specific requirements throughout different developmental stages and that sex-biased expression at different organs might facilitate the operation of sex-specific traits. Finally, we find evidence that dosage compensation is conserved in the *Heliconius* genus, a fact that suggests that once dosage compensation evolves in a particular system, it is more likely to stay conserved despite mating system type or other factors that might influence the evolution of dosage compensation.

Results and Discussion

Sex-Biased Gene Expression

To improve our understanding of how sex-biased gene expression in combined eye and brain tissue of *Heliconius* butterflies evolves, we analyzed whole transcriptomes from five *Heliconius* butterflies: *H. charithonia*, *H. sara*, *H. erato*, *H. doris*, and *H. melpomene* (fig. 1). We generated *de novo* Trinity assemblies for each species. *De novo* transcriptomes had from 31,103 to 40,275 assembled genes with N50s ranging from 972 to 1,075 bp (supplementary table S1, Supplementary Material online). On an average, 75–85% of the reads mapped to each corresponding species transcriptome (supplementary table S2, Supplementary Material online). A one-factor analysis was done using EdgeR and a false discovery rate (FDR) of 5% was applied to identify sex-biased genes (supplementary tables S3–S8, Supplementary Material online). We found that the degree of sexual dimorphism present in eye and brain gene expression varied across *Heliconius* (fig. 1). The species showing the highest number of sex-biased genes was *H. erato*, with 285 sex-biased genes, an amount that contrasted with the 39 sex-biased genes identified in *H. melpomene*. To ensure that the number of sex-biased genes detected in each species is not dependent on the gene expression variance within replicates, we checked for the presence of a correlation between the number of sex-biased genes detected and the within sample variance. We found no correlation, (supplementary table S3 and fig. S1, Supplementary Material online, Spearman correlation,

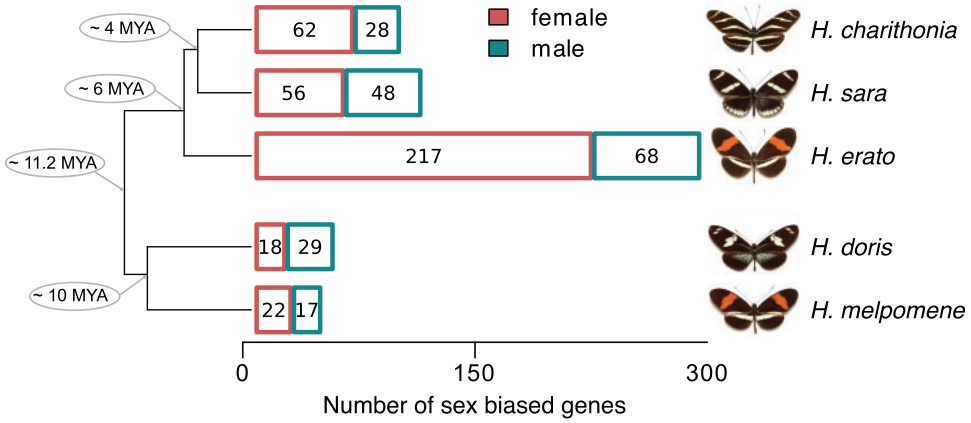

Fig. 1. Sex-biased gene expression in combined eye and brain tissue across *Heliconius* butterflies. Horizontal bars show the number of sex-biased genes identified in each species. Red outline corresponds to the number of female-biased genes and blue outline to the number of male-biased genes. Divergence times and phylogenetic relationships of *Heliconius* species are from Kozak et al. (2015).

rho = 0.3, *P* value = 0.683), thus ensuring that the number of sex-biased genes detected is not being affected by the sample variance.

All the *Heliconius* species analyzed in this study, with the exception of *H. doris*, had a higher number of female-biased genes (supplementary fig. S2, Supplementary Material online), and this difference was significant in *H. charithonia* (sign test, *P* = 4.38e-5) and *H. erato* (sign test, *P* = 1.71e-14). In contrast, *H. doris* had more male-biased (n = 29) than female-biased genes (n = 18), although this difference was not significant (sign test, *P* = 0.1439). These results are in agreement with those found in other lepidopterans like in *M. sexta* and *Bicyclus anynana* heads, where ~70% of the sex-biased genes identified showed female-biased expression (Smith et al. 2014; Macias-Muñoz et al. 2016). The proportion of female- or male-biased genes found is highly variable according to the species, the developmental stage or the organ tested (Grath and Parsch 2016). For example, in brain and head tissue from flies and birds, males were found to have a higher proportion of sex-biased genes (Catalán et al. 2012; Mueller et al. 2016), whereas in fish heads and bodies, more female-biased genes were found (Yang et al. 2016). On the other hand, in mosquitos, gonads had a higher number of female-biased genes, a result that contrasts with other studies done in nematodes, flies, and primates where gonads have a higher number of male-biased genes (Parisi et al. 2003; Khaitovich 2005; Albritton et al. 2014). The change of sex-biased expression across phylogenies, organs, and developmental stages is thought to be strongly correlated with the strength of selection being experienced by each sex, shifting sex expression optima accordingly (Zhang et al. 2007; Harrison et al. 2015). In the case of *Heliconius* brain and eye tissue more female-biased genes were found in most species surveyed, suggesting that female-specific natural selection is stronger in eye and brain tissue than in males.

TransDecoder was used to predict putative coding regions in each transcriptome and on an average 30% of the assembled transcripts had at least one predicted ORF (open reading frame), with approximately seven ORFs per transcript. Gene annotation was done by doing a blastp homologous search with Flybase, UniProt, and Pfam databases, resulting in 48–54% annotation success across the five species (Dryad data identifier: doi:10.5061/dryad.ds21fv5). From all annotated transcripts, 3,886 have the same annotation hit across all species and 1,088–1,745 have a unique annotation in each species (fig. 2A). When looking only at sex-biased genes, 66–85% had an annotation hit (supplementary tables S9–S13, Supplementary Material online). There was little overlap of annotated sex-biased genes across species (fig. 2B and supplementary table S14, Supplementary Material online). When orthologous relationships were assessed across our five *Heliconius* species, 2,393 orthologous gene groups were identified (supplementary table S15, Supplementary Material online). From the ortho-groups identified, only two clusters shared sex-biased expressed genes across species (supplementary table S15, Supplementary Material online). The ortho-group 325_1 had sex-biased orthologs in *H. charithonia* and *H. sara*. Genes belonging to the ortho-group 325_1 have functions in actin binding, salivary gland histolysis, wound healing, and cell spreading (Chintapalli et al. 2007). The second ortho-group with shared sex-biased genes was the ortho-group 69_0, with sex-biased genes in *H. sara* and *H. erato*, and functions related to RNA binding (Chintapalli et al. 2007). We observed a significant underrepresentation of shared sex-biased genes in the ortho-groups identified (Fisher's exact test, *P* value < 0.0001) as well as in the number of shared annotation hits across species (Fisher's exact test, *P* value < 0.0001) when compared with transcriptome-wide expectations.

The lack of overlap of sex-biased genes across species might be due to drift acting on sex-biased expression patterns causing expression to evolve away and independently from an optimal level shared across species. On the other hand, sex- and species-specific directional selection could also be the cause of most of the sex-biased genes being expressed in a species-specific manner. To explore under which evolutionary forces sex-biased genes might be evolving, we calculated the deltaX (Δx) (supplementary Appendix S1, Supplementary Material online) statistic as a measurement of the strength of gene expression divergence to standing gene expression

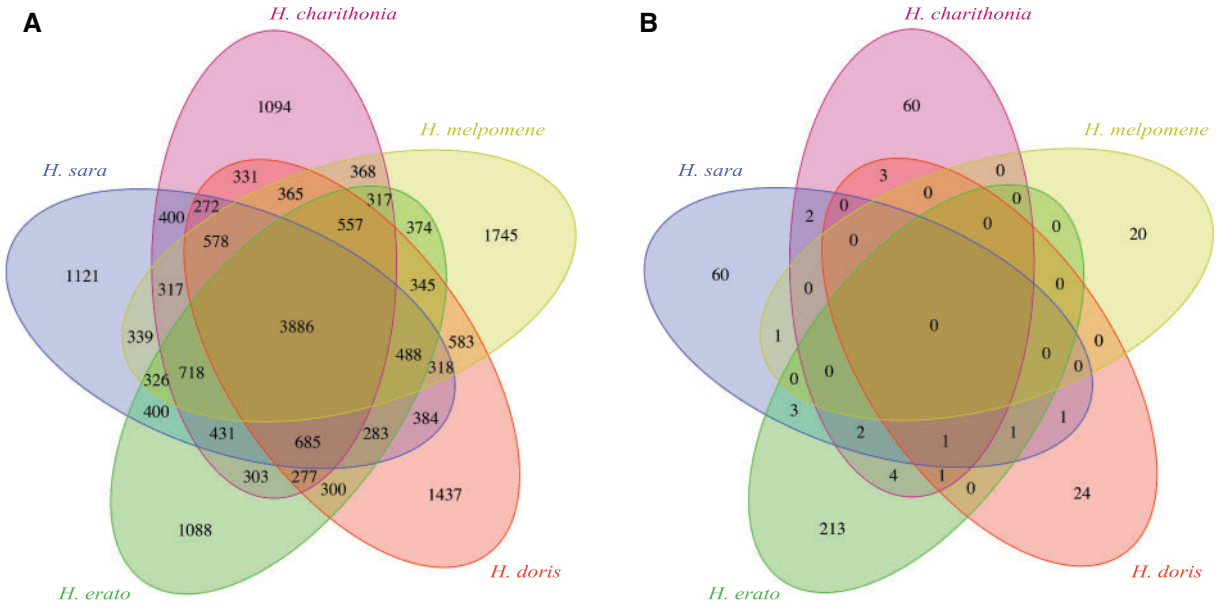

Fig. 2. Venn diagram showing the overlapping annotated genes between five *Heliconius* species. (*A*) Overlapping regions indicate shared genes among species for whole transcriptomes. (*B*) Overlapping sex-biased genes.

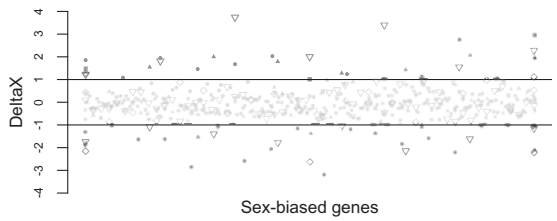

Fig. 3. Gene expression divergence of sex-biased genes in *Heliconius* butterflies. Δx (*y* axis) was calculated for all sex-biased genes as a measure of gene expression divergence for: ▽ *H. charithonia*, ▲ *H. sara*, ● *H. erato*, ■ *H. melpomene* and ◇ *H. doris*. Genes with $\Delta x > 1$ and $\Delta x < -1$, threshold-marked with solid black lines, are likely to be experiencing directional selection for gene expression.

variation. Δx assesses expression level divergence between two closely related species in relation to the variation in expression levels within a species of interest, a test analogous to the McDonald–Kreitman test (McDonald and Kreitman 1991; Moghadam et al. 2012; Dean et al. 2015). When $\Delta x > 1$ or $\Delta x < -1$, gene expression divergence exceeds gene expression variation within species, suggesting that directional selection is acting on gene expression levels (Moghadam et al. 2012). In our five *Heliconius* species, 68 out of 565 (*H. charithonia*: 15, *H. sara*: 13, *H. erato*: 31, *H. melpomene*: 3 and *H. doris*: 6) sex-biased genes are most likely evolving under positive selection, causing gene expression levels to be under- or overexpressed due to directional selection instead of drift (fig. 3). These results show that even though most sex-biased genes are probably evolving under drift, a fraction of these are experiencing directional selection, pushing gene expression levels toward a sex-specific expression optima. Shifts toward a sex specific optima of gene expression have been reported in nature like in the Malawian cichlid fish or as a product of artificial selection toward sex-specific traits like in chicken (Roberts et al. 2009; Khabbazian et al. 2016).

From all the genes identified as sex-biased, only two were sex-biased among *H. charithonia*, *H. sara*, and *H. erato*: *UVRh1* and *CG9492*, the latter encoding a dynein heavy chain protein, and the former encoding the UV1 opsin. Another gene whose sex-biased expression is shared among some species is the transcription factor *Krueppel homolog 1* (*Kr-h1*), which in *H. sara*, *H. erato*, and *H. doris* shows a 2- to 3-fold male-biased expression. In *H. charithonia*, *Kr-h1* also shows a 2-fold male-biased expression (supplementary table S4, Supplementary Material online) but this difference is not significant (*P* value = 0.06). In *D. melanogaster*, overexpression of *Kr-h1* affects ommatidia photoreceptor maturation, suggesting that a repression of *Kr-h1* is necessary for normal photoreceptor development (Fichelson et al. 2012). In the case of *CG9492*, it is expressed in *D. melanogaster*'s Johnston's organ (Senthilan et al. 2012), as well as in the brain, where it has a role during dendrite development (Wang et al. 2015). Studies of *CG9492* have found that a Krueppel-like transcription factor plays a role in its regulation (Wang et al. 2015). Besides *Kr-h1* having a role in normal photoreceptor development (Fichelson et al. 2012), in thrips and in the beetle *Tribolium castaneum Kr-h1* is involved in mediating metamorphosis (Minakuchi et al. 2009; Minakuchi et al. 2011), whereas in the honeybee, increased levels of *Kr-h1* are associated with the transition from nursing to foraging behavior (Grozinger et al. 2003). These findings in *Drosophila* beg the question as to whether an interaction between *CG9492* and *Kr-h1* is present in *Heliconius*, and whether these two genes have a role in the regulation of sex-biased opsin expression.

The sex-biased genes showing the most differential expression are shown in figure 4. In *H. charithonia*, *H. sara*, and *H. erato*, *UVRh1* is strongly female-biased. Read counts for *UVRh1* in males averaged 6 raw reads, whereas in females

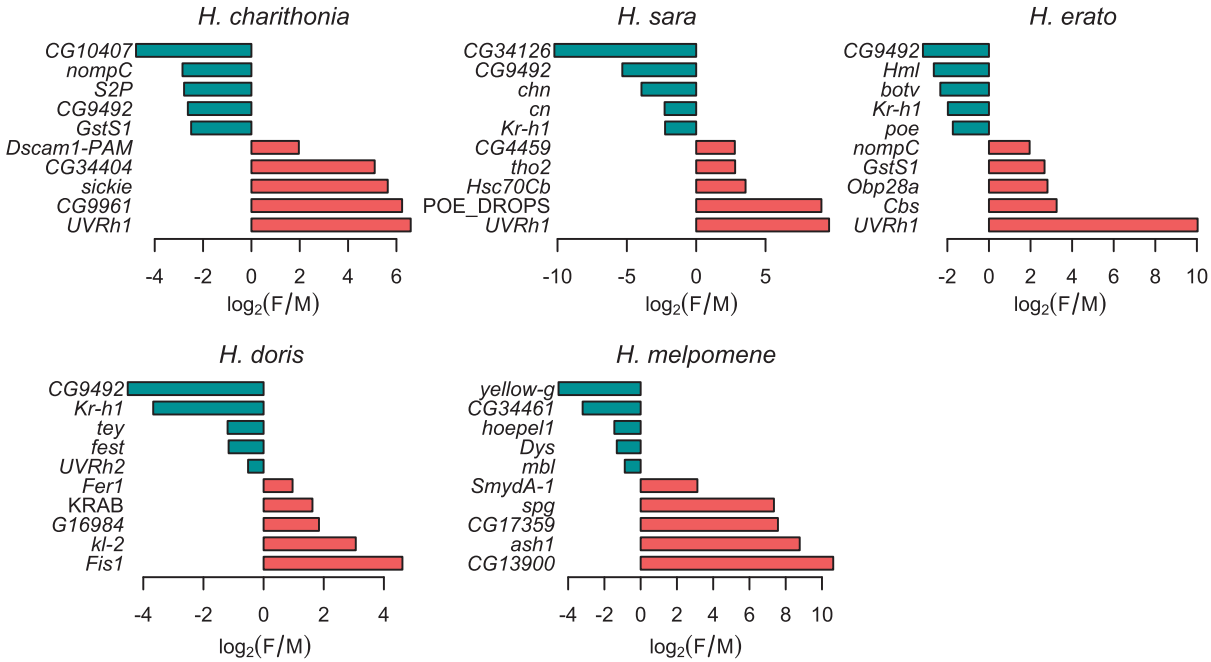

Fig. 4. Annotated genes showing the strongest sex-biased expression. Fold differential expression between females (F) and males (M) in five *Heliconius* butterflies. The first five genes, from top to bottom, are significantly overexpressed in males (blue) and the last five genes are overexpressed in females (red).

the average read count was 2,270 showing an almost female-specific *UVRh1* expression. The fact that *UVRh1* shows female-specific expression implies that the expression of *UVRh1* is detrimental in males. *UVRh1* and *UVRh2* have different UV light absorption maxima with UV1 rhodopsin peaking at 355 nm and UV2 peaking at 390 nm (McCulloch et al. 2016). Theoretical models suggest that males benefit from expressing only UV2 in visual discrimination of different shades of yellow while females benefit from expressing UV1 and UV2 in discrimination of different shades of ultraviolet (see table 3 in Finkbeiner et al. 2017). The paralogs *UVRh1* and *UVRh2* could be an example of the resolution of sexual antagonism (Wright and Mank 2013), achieved by a duplication event, followed by subfunctionalization and female-specific expression of *UVRh1*, probable driven by female-specific selection for color vision in the UV light spectrum and possibly male-specific selection for discriminating between different shades of yellow, a color found on many *Heliconius* wings.

A female-specific expression of *UVRh1* might be linked to the female-specific behavior of finding suitable oviposition sites. Variation in leaf color and brightness are cues that are used by various butterflies to assess quality and suitability of oviposition hosts (Green et al. 2015). As some *Passiflora* leaves reflect in the UV-green (Briscoe, personal observation), it maybe important for females to have UV color vision. In *H. charithonia*, larvae reflect under UV light, a cue that could be used by females to assess larval density and competition during foraging for oviposition sites (Estrada 2009). UV reflectance could also serve as a cue during courtship behavior (Finkbeiner et al. 2017). In the butterflies *Colias eurytheme* (Pieridae) and *Bicyclus anynana* (Nymphalidae), UV reflectance on male wings affects female choice (Robertson and Monteiro 2005; Papke et al. 2007), making wing UV reflectance a putative signal for assessing male suitability and fitness. Rigorous behavior experiments coupled with genetic ones have to be done in order to test the hypothesis that the sex-biased expression found in the UV opsin is linked to a female- or male-specific behavior.

Some of the sex-biased genes which show sex-biased expression only in a specific species, with vision and brain function related annotations, include the transcription factor *charlatan* (*chn*) (*H. sara*) involved in phototransduction and eye development, *vacuolar peduncle* (*van*) (*H. doris*) involved in mushroom body development, *photoreceptor hydrogenase* (*Pdh*) (*H. charithonia*), which has a role in retinal metabolic processes, *muscleblind* (*mbl*) (*H. melpomene*) involved in eye and rhabdomere development, and *Rab32* (*H. erato*) involved in ommochrome biosynthetic process (see supplementary tables S9–S13, Supplementary Material online, for full annotation of sex-biased genes). In other organisms, a low overlap of sex-biased genes across species has also been found; specifically from the occipital cortex in human, macaques, and marmosets (Reinius et al. 2008) and in birds from gonads and spleen across six bird species (Harrison et al. 2015).

Evolutionary Rates of Sex-Biased Genes

Females and males experience different selective pressures, which can lead to sex differences in gene expression. In several species there is evidence that protein coding genes with sex-biased expression show accelerated rates of evolution, a trend that generally is accentuated in male-biased genes expressed in male-specific tissues (Ranz et al. 2003; Zhang et al. 2004; Meisel 2011). If sex-biased genes emerged because of

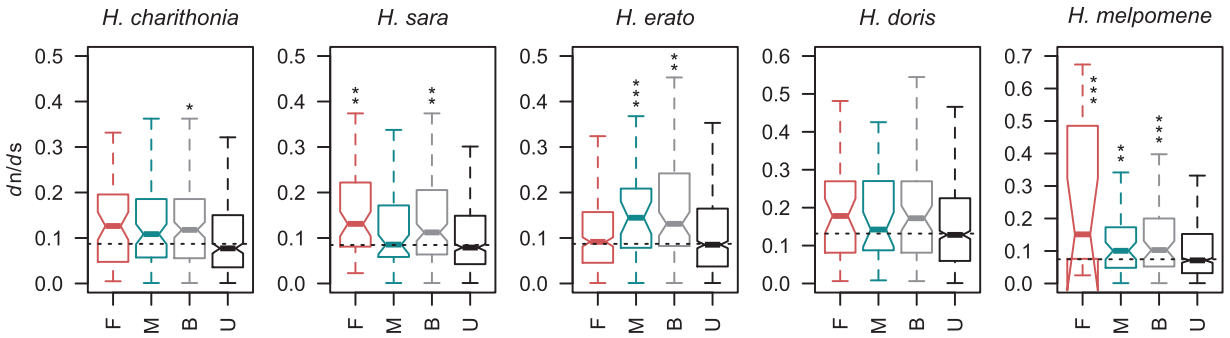

Fig. 5. Evolutionary rates of sex-biased and unbiased genes. Boxplots show d_N/d_S distribution for female-biased (F), male-biased (M), all sex-biased (B) and unbiased (U) genes. Dashed horizontal line indicates d_N/d_S median for all genes analyzed for each species. Significance was determined by a generalized linear model. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

sex-specific selective pressures or sexual selection, higher evolutionary rates might be expected in sex-biased compared with unbiased genes. By quantifying the rate of nonsynonymous (d_N) versus synonymous (d_S) replacements along a protein sequence (d_N/d_S) we can get an idea of a proteins' evolutionary pace (Kimura 1984; Yang 2007). Thus, we calculated d_N/d_S for the sex-biased genes identified and for ~ 500 randomly chosen unbiased genes for each species independently (fig. 5).

When comparing d_N/d_S of sex-biased and unbiased genes, we observed that sex-biased genes had a significantly higher d_N/d_S (supplementary table S16 and Appendix S2, Supplementary Material online). For *H. charithonia*, *H. sara*, *H. doris*, and *H. melpomene* female-biased genes showed 27–53% higher d_N/d_S than unbiased genes; this difference was significant after applying a generalized linear model in *H. sara* and *H. melpomene*. *Heliconius erato* only showed a significant increase of d_N/d_S in male-biased genes with 58% higher rates. Higher d_N/d_S in sex-biased genes can be explained by positive selection causing acceleration of evolutionary rates, by relaxation of purifying selection or by codon bias usage (Zhang et al. 2004; Hambuch and Parsch 2005). If d_N/d_S is higher in sex-biased genes because of codon bias one would expect d_S rates to be lower in sex-biased relative to unbiased genes because selective constraints at synonymous sites would decrease synonymous codon replacements. To test if this is the case, we first calculated codon bias by calculating a deviation of the effective number of codons used in sex- versus unbiased genes as measured by Nc (Wright 1990) and implemented in ENCprime (Novembre 2002). The Nc statistic measures how codon usage for a gene might depart from equal usage of synonymous codons. We found that the distribution of codon usage as quantified by Nc is equal in sex- and unbiased genes in *Heliconius* as tested by a Wilcoxon test (*P* values: *H. charithonia:* 0.74, *H. sara:* 0.07, *H. erato*: 0.65, *H. melpomene*: 0.5, *H. doris*: 0.37) (supplementary fig. S3, Supplementary Material online). Next, we looked for a significant decrease in d_S in sex-biased genes when compared with unbiased genes and found no significant difference, as would be expected in the presence of strong codon bias (table 1). Unlike d_S, we do observe a significant difference of d_N between sex- and unbiased genes. Sex-biased genes have higher d_N than unbiased genes, a result indicating that higher d_N/d_S in sex-biased genes is driven by an increase of d_N instead of a decrease of d_S.

Faster evolutionary rates of female-biased genes in brain tissue have also been reported in birds (Mank et al. 2007), a result that contrasts with the higher evolutionary rate usually found in male-biased genes, especially when analyzing reproductive tissue, but also in somatic tissue (Meiklejohn et al. 2003; Zhang et al. 2004; Mank et al. 2008). For female- and male-specific life history requirements, nonreproductive organs might be used in a sex-biased manner. Some organs might have a more important role for one sex than for the other; a fact that might be reflected in the amount of sex-specific positively selected genes. The higher d_N/d_S found in sex-biased genes could be the result of a relaxation of purifying selection or an increase in positively selected substitutions (Ellegren and Parsch 2007). Since we observed an increase of d_N but not a decrease of d_S, we suggest that the inflated d_N/d_S observed in sex-biased genes may be a signal of positive selection. Further population genetic data would be needed to explore the evolutionary forces shaping sex-biased gene expression in *Heliconius* butterflies.

Dosage Compensation

Currently, dosage compensation has only been addressed in the *melpomene/cydno* clade (Walters et al. 2015), giving us the opportunity to assess if dosage compensation is a trait present throughout the *Heliconius* phylogeny. We looked for evidence of dosage compensation in four *Heliconius* species: *H. charithonia*, *H. sara*, *H. erato*, and *H. doris*. These four species belong to three different phylogenetic clades, which include the *sara/sapho* clade (*H. charithonia*, *H. sara*), the *erato* clade (*H. erato*), and the *doris* clade (*H. doris*). We analyzed and compared gene expression levels between females (ZW) and males (ZZ) in different gene expression categories, and between the autosomes and the Z-chromosome within each sex to assess dosage compensation in eye and brain tissues.

Data Filtering

The filtering criteria applied to expression data might affect the conclusion of whether dosage compensation is present or not, as illustrated by Jue et al. (2013) and Castagné et al. (2011). Here, we explored two types of data filtering and processing in *Heliconius*. Firstly, we applied three

Table 1. Median d_N and d_S Rates for Female-Biased, Male-Biased, and Unbiased Genes in Five *Heliconius* Species.

	d_N			*P* value	
Species	female-biased	male-biased	unbiased	F–U[a]	M–U[b]
H. charithonia	0.0408 (0.0230–0.0562)	0.0264 (0.0200–0.0376)	0.0209 (0.0167–0.0252)	0.0002	0.0447
H. sara	0.0350 (0.03020–0.0427)	0.0319 (0.0240–0.0443)	0.0214 (0.0190–0.0246)	0.0005	0.0012
H. erato	0.0213 (0.0189–0.0253)	0.034 (0.0249–0.0512)	0.0209 (0.0186–0.0239)	0.7702	0.0920
H. doris	0.0368 (0.0226–0.0511)	0.0399 (0.0223–0.0607)	0.0294 (0.0267–0.0341)	0.4741	0.2495
H. melpomene	0.0406 (0.0122–0.0553)	0.01865 (0.0143–0.0275)	0.0154 (0.0133–0.0173)	0.0032	0.0566
	d_S			*P* value	
Species	female-biased	male-biased	unbiased	F–U[a]	M–U[b]
H. charithonia	0.3318 (0.2932–0.3745)	0.2631 (0.2129–0.2962)	0.2626 (0.2512–0.2846)	1.861e-05	0.5184
H. sara	0.2661 (0.2252–0.3015)	0.3664 (0.3259–0.4091)	0.2711 (0.2589–0.2900)	0.2491	2.636e-06
H. erato	0.2532 (0.2347–0.2637)	0.245 (0.2201–0.2977)	0.2608 (0.2524–0.2705)	0.0535	0.5684
H. doris	0.2255 (0.1774–0.2763)	0.2384 (0.2184–0.3055)	0.2466 (0.2369–0.2616)	0.1375	0.6248
H. melpomene	0.18455 (0.1174–0.3174)	0.22065 (0.1671–0.2470)	0.2084 (0.1727–0.2505)	0.5502	0.7231

NOTE.—Significance in d_N and d_S between sex-biased and unbiased genes was tested using a Wilcoxon test. In parenthesis, 95% confidence intervals of median values are shown.
[a]Wilcoxon test's *P* value, female-biased (F) versus unbiased genes (U).
[b]Wilcoxon test's *P* value, male-biased (M) versus unbiased genes (U).

hard-thresholds on the expression levels, where genes were considered to be expressed if they have higher expression values than 1, 3, and 5 FKPM. This approach has been commonly used to assess dosage compensation (Smith et al. 2014; Mahajan and Bachtrog 2015). When utilizing thresholds of 1, 3, and 5 FPKM to define expressed genes, we observe that the higher the filtering threshold the more shifted the expression distribution becomes toward "highly" expressed genes (supplementary fig. S4, Supplementary Material online), causing a disproportionate compression of the expression values. At a filtering threshold of 5 FPKM ~40% of the Z-linked genes are removed, causing the exclusion of a high number of lowly expressed genes for subsequent analysis (supplementary table S17, Supplementary Material online). As observed in our analysis (supplementary figs. S4–S7 and table S18, Supplementary Material online), this can shift the mean and median chromosomal expression values causing also a shift in the degree of dosage compensation. To avoid an overtruncation of the expression data and an arbitrary selection of a threshold, we explored a second approach to set up a minimum expression threshold, that is, characterizing the observed $\log_2$ FPKM expression distributions by using a Kolmorogov–Smirnov statistic and multivariate adaptive regression splines as described in the data-adaptive flag method (DAFS) (George and Chang 2014). Using this method on *H. charithonia*, *H. sara*, *H. erato*, and *H. doris* expression values, we found minimum expression thresholds ranging from 0.14 to 0.18 or 1.10 to 1.13 for FPKMs that were not $\log_2$ transformed. Using these threshold values, we proceed to investigate dosage compensation.

Z-Chromosome to Autosomes Expression

Firstly, we checked for differences in expression levels in the Z-chromosome versus the autosomes in females (Z:AA) and males (ZZ:AA) (fig. 6). We found that in both females and males, the autosomes showed higher median expression when compared with the Z-chromosome, with this difference always being stronger in females. Females showed 10–16%, and males 6–14% higher median expression in the autosomes than in the Z (fig. 6). In females higher autosomal expression was significant in all species, and in males only in *H. charithonia* was this difference not significant (supplementary table S17, Supplementary Material online). We also observed that the Z-chromosome has similar expression levels between females and males, with the exception of *H. erato*, where the female's Z has significantly lower expression than the male's Z (fig. 6). When we looked at the (Z:A) expression ratios drawn from median chromosomal expression in females and males, we found that in males Z:A ranged from 0.85 in *H. sara* to 0.93 in *H. charithonia*, whereas for females Z:A ranged from 0.83 in *H. sara* to 0.89 in *H. charithonia* (fig. 7). In humans and mice this pattern was also observed in somatic dosage compensated tissue, where X:A ratios were always higher in the homogametic sex (Deng et al. 2011).

A lower Z-to-autosome expression has been reported in other lepidopterans (Huylmans et al. 2017) including in *H. melpomene* (Walters et al. 2015), as well as in birds (Wang et al. 2017) and mammals (Julien et al. 2012; Jue et al. 2013). In *Heliconius* these results could be explained by the presence of a mechanism down-regulating the Z-chromosome, similar to the one present in *B. mori*, where an epigenetic mechanism for dosage compensation was found and which consists in down regulating one of the male's Z chromosomes by the *masculinizing* zinc-finger protein *masc* (Kiuchi et al. 2014). The down-regulation of one of the male's Z chromosomes would equalize the sex chromosome expression between females and males but would also cause an expression imbalance between the Z and the autosomes.

Female Versus Male Chromosomal Expression

In the presence of dosage compensation, an expression balance between the single female's Z and the two male's Z is expected. Therefore, we further examined female versus male (F:M) expression level differences in the autosomes (AA:AA)

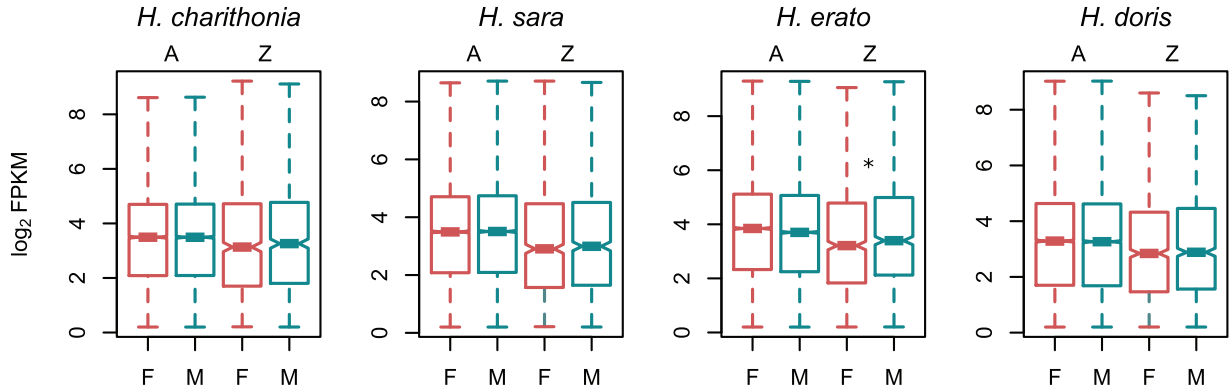

Fig. 6. Whole transcriptome chromosomal expression in female and male *Heliconius*. Boxplots show the distribution of normalized expression levels $\log_2$ (FPKM) in female (F) and male (M) Z-chromosome (Z) and autosomes (A). Significance was determined by a Wilcoxon test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

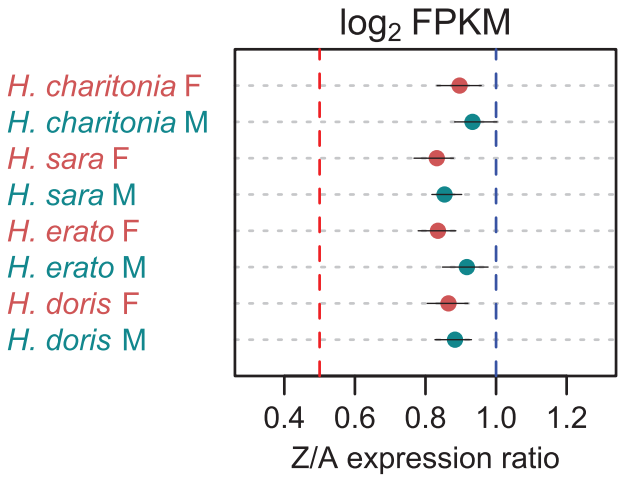

Fig. 7. Median Z-chromosome versus autosome expression ratios for female and male *Heliconius* butterflies. Dots indicate median Z-chromosome/Autosome (Z:A) expression ratio. Horizontal solid lines represent 95% confidence intervals drawn from 10,000 bootstrap samples from female and male expression levels. Blue lines indicate an expression ratio Z:A = 1, and red lines indicates an expression ratio Z:A= 0.5. F =female, M = male.

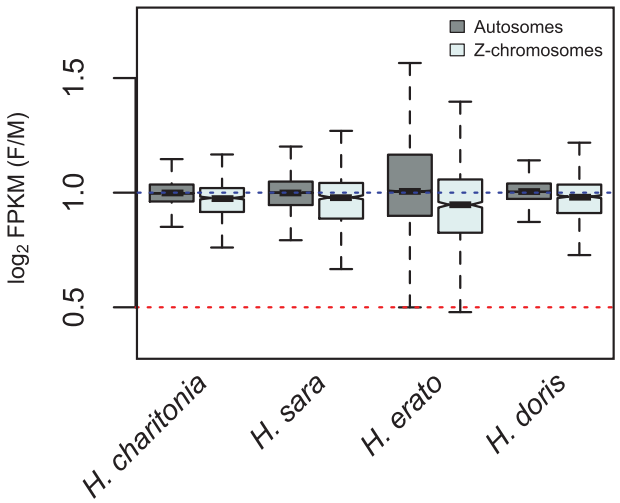

Fig. 8. Distribution of median female to male expression ratios in the autosomes and the Z-chromosome. Female/male (F:M) expression ratios drawn per gene expressed in the autosomes (dark gray) and the Z-chromosome (light gray) for four *Heliconius* species. The *x* axis shows the four *Heliconius* species tested. Blue horizontal line indicates F:M = 1 and red line F:M = 0.5.

and the Z-chromosome (Z:ZZ). For the autosomes, F:M ratios range from 0.99 in *H. charithonia* to 1.005 in *H. erato*, whereas in the Z-chromosome F:M median expression ratios range from 0.95 in *H. erato* to 0.98 in *H. doris* (fig. 8). When tested for equal expression of the autosomes and the Z using F:M ratios with a two-sample Wilcoxon test, we found that for all the species the autosomes and the Z-chromosome differ significantly in their F:M expression ratios (*P* values: *H. charithonia*: 1.08e-17, *H. sara* 1.25e-10, *H. erato*: 5.90e-22, *H. doris*: 4.35e-19). These data suggest that the Z-chromosome in the heterogametic sex undergoes dosage compensation, but that dosage compensation might not be global. In *Drosophila*, variation has been found in the effect of gene dosage across genes, showing that some genes and gene networks are more sensitive to dosage effects than others (Lee et al. 2016). For *Heliconius*, a similar scenario in which genes escaping dosage compensation have no or only a mild effect to their gene networks is plausible.

To further uncover female and male expression level differences in the Z-chromosome, we partitioned its expression into four quantiles (fig. 9). For most of the species females have significantly lower expression in comparison to males for lowly-to-medium expressed genes (fig. 9). Even though significance was detected by a Wilcoxon test, the difference between female and male Z expression in the low-to-medium expressed genes was only slightly >1-fold (1.06-1.2) (supplementary table S19, Supplementary Material online). The highest expressed gene category (Q4) shows no difference between females and males in any of the species (fig. 9). These data suggest that for Z-linked genes with low-to-medium expression, females tend to have a slight but significantly lower expression in comparison to males, revealing that these gene categories might be more prone to escape dosage compensation.

Our analysis of four *Heliconius* species representing four different phylogenetic clades suggests the presence of dosage

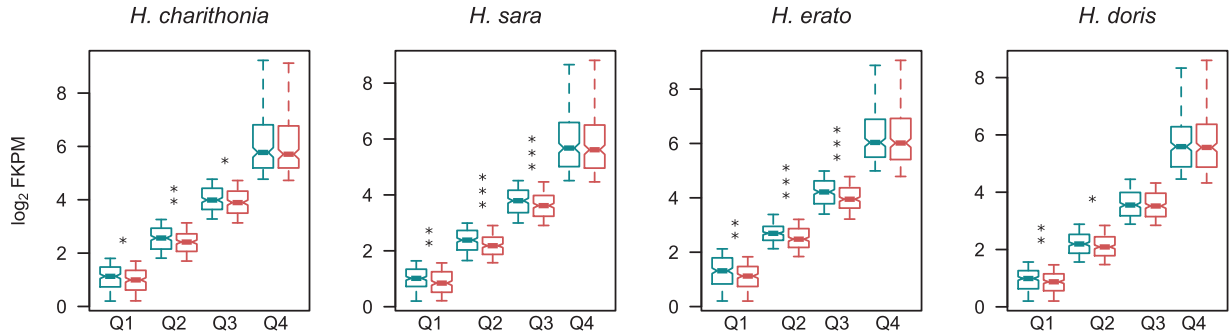

FIG. 9. Z-chromosome expression in male and female *Heliconius* butterflies. Boxplots show the distribution of $\log_2$ transformed FPKM expression values. Horizontal solid line indicates chromosomal median expression level. Expression data was partitioned in four expression quartiles (Q1–Q4), with the first quartile containing the genes with the lowest expression levels and the fourth quartile containing the genes with the highest expression. Significance between female and male Z expression was determined by a Wilcoxon test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Blue indicates males and red indicates females.

compensation in somatic tissue in the whole *Heliconius* group, similarly to what has been reported in other lepidopteran species (Kiuchi et al. 2014; Walters et al. 2015; Huylmans et al. 2017). It has been hypothesized that the mating system type might influence the development of a dosage compensation mechanism. For example, in ZW systems where males are experiencing a higher selective pressure and have a higher fitness success when compared with females, the Z's Ne is $< 3/4$ the Ne of the autosomes, a situation that would hinder the development of a dosage compensation mechanism (Wright and Mank 2013; Mullon et al. 2015). In *Heliconius*, both polyandrous and a monoandrous mating systems can be found. A single mating occurs in females of species where males exhibit a pupal guarding behavior, whereas nonpupal maters mate several times (Beltrán et al. 2007). According to spermatophore counts, *H. erato*, *H. charithonia*, and *H. sara* females mate only once. *Heliconius melpomene* and *H. doris* mate several times, as females had several spermatophores (Walters et al. 2012). Despite the different mating systems found in *Heliconius* that would affect the Ne of the Z and thus the strength of genetic drift acting on it, dosage compensation has stayed conserved in the whole clade. The presence of dosage compensation in lepidopterans contrasts with the lack of dosage compensation found in birds (Itoh et al. 2007), which also have a ZW system and diverse mating systems. The level of gene–gene interactions between the Z and the autosomes might be a factor promoting the evolution of dosage compensation, with clades with high Z-to-autosome interactions being under a higher pressure of developing a dosage compensation mechanism. The mechanism by which *Heliconius* achieves dosage compensation still needs to be elucidated. From our data, a global dosage compensation mechanism could be present like one present in *B. mori* or *Drosophila* (Gelbart and Kuroda 2009; Kiuchi et al. 2014) but a scenario where genes are compensated individually depending on their dosage sensitivity and gene-to-gene interactions should not be discarded.

Genomic Distribution of Sex-Biased Genes

In many species, including insects and vertebrates, it has been observed that sex-biased genes are not randomly distributed across the genome, as they are often either enriched or depleted from the sex chromosomes (Kaiser and Ellegren 2006; Albritton et al. 2014). The cause of enrichment of sex-biased genes on the sex chromosomes might be different for every species. Natural selection on sex-biased genes, differing sex chromosome number between sexes or the presence or absence of dosage compensation, could potentially bias the distribution of sex-biased genes in the genome (Ellegren and Parsch 2007).

To investigate whether sex-biased genes in *Heliconius* have a skewed genomic distribution, we used *H. melpomene* chromosomal annotation to infer chromosomal location of sex- and unbiased genes in *H. charithonia*, *H. sara*, *H. erato*, and *H. doris*. This is a reasonable approach since there is a high conservation of chromosome synteny within Lepidoptera (Pringle et al. 2007; Dasmahapatra et al. 2012). In *H. charithonia* and *H. doris*, we find a significant Z-chromosome enrichment in both female- and male-biased genes (fig. 10). In the case of *H. erato* and *H. sara* only male-biased genes are significantly enriched in the Z-chromosome and in *H. melpomene* no significant enrichment was found. Assuming that sex-biased gene expression is the result of resolving sexual antagonism, it is predicted that recessive mutations advantageous for the heterogametic sex will be enriched on the Z (Rice 1984; Oliver and Parisi 2004). When recessive mutations are female beneficial but male detrimental, these will be immediately exposed for natural selection to act on them in the heterogametic sex, but in a heterozygous state these will be covered in the homogametic sex, leading to an enrichment of female beneficial mutations on the Z (Rice 1984; Charlesworth et al. 1987; Vicoso and Charlesworth 2006). Such a scenario could explain the enrichment of female-biased genes observed in *H. charithonia* and *H. doris* (fig. 10).

The enrichment of male-biased genes observed in the Z of *H. charithonia*, *H. sara*, *H. erato*, and *H. doris* could be driven by male beneficial dominant Z-linked mutations. In this scenario, directional selection would act on these alleles 2/3 more often than for female detrimental mutations, which when sitting on the Z would be selected against only 1/3 of the time, allowing for an accumulation of male-biased genes on the Z (Rice 1984; Charlesworth et al. 1987; Vicoso and

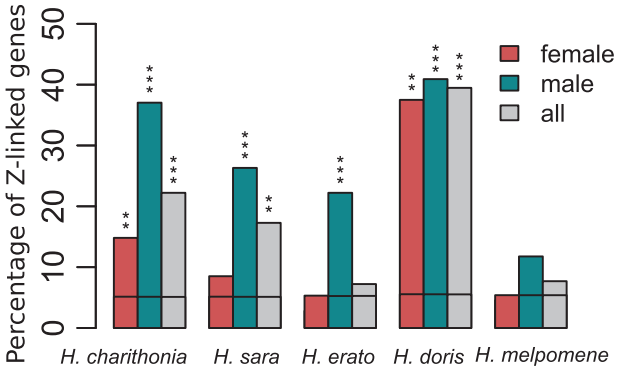

Fig. 10. Chromosomal enrichment analysis of sex-biased genes. Percentage of sex-biased genes located in the Z-chromosome (vertical bars) and in the autosomes (horizontal black solid lines). Significance was determined by a Fisher's exact test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Charlesworth 2006). On the other hand, dosage overcompensation of the Z could also lead to an enrichment of sex-biased genes. This has been observed in head and brain of *Drosophila*, where sex-biased genes were found to be enriched on the X and whose position relative to the dosage compensation machinery was correlated with their level of sex-biased expression. Hence it was concluded that those sex-biased genes closest to the dosage compensation machinery might suffer overcompensation (Chang et al. 2011; Catalán et al. 2012; Meisel et al. 2012). In birds, which like butterflies have a ZW chromosome system, male-biased but not female-biased genes from gonad and brain were overrepresented on the Z chromosome. This enrichment could be explained by a resolution of sexual antagonism but also by the fact that dosage compensation in birds appears to be absent, leading to an enrichment of male-biased genes only (Kaiser and Ellegren 2006; Storchová and Divina 2006; Ellegren et al. 2007). In *Heliconius* butterflies, an enrichment of male-biased genes on the Z could then also be explained by some genes in the Z escaping dosage or by Z overcompensation. If this is the case, we could look for enrichment on the Z in genes that have at least 1.5-fold differential expression to identify those genes showing sex-biased expression regardless of a dosage compensation effect. Only five genes had <1.5-fold expression difference (one in *H. charithonia*, two in *H. erato* and two in *H. doris*) indicating that an enrichment of the Z caused by escaping compensation is unlikely.

Conclusion

The five *Heliconius* species analyzed in this study belong to five major clades in the *Heliconius* phylogeny, spanning divergence times from ~4 to 12 Ma (Kozak et al. 2015). The degree of sexually dimorphic gene expression in combined eye and brain tissues varies across our *Heliconius* species, where we observed differences of up to 7-fold in the number of sex-biased genes found. Our results suggest that females and males have species-specific eye and brain usage, probably adjusted to their sex-specific life history requirements. We also observed that sex-biased genes have higher evolutionary rates than unbiased genes and that in some *Heliconius* species female-biased genes and in other species male-biased genes are driving this difference. This might reflect differences in the selective pressures that females and males are experiencing, which also seems to be a species-specific phenomenon. From all the sex-biased genes that we identified, only a few were shared among *Heliconius*. These shared genes could form part of a conserved shared regulatory network in the visual system. Nevertheless, the majority of sex-biased genes are not shared between species suggesting that sexual selection might have driven sexually dimorphic gene expression. We also find evidence of dosage compensation in the five examined *Heliconius* species, thus showing that dosage compensation is putatively present in the whole the *Heliconius* clade. Finally, we find that sex-biased genes are not randomly distributed in the genome but are enriched in the Z-chromosome, a phenomenon that could be explained by a Z selective advantage and/or by the presence of a dosage effect.

Materials and Methods

Samples and RNA-Sequencing

Pupae from *H. charithonia*, *H. sara*, *H. erato*, *H. doris*, and *H. melpomene* were obtained from The Butterfly Farm—Costa Rica Entomological Supply. One to three days after eclosion, butterflies were fresh frozen at −80°C until RNA extraction. Combined eye and brain tissue were dissected by removing antennae, palps, and proboscis from the head and RNA was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. RNA was purified using a NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA). Purified RNA was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) and RNA integrity was checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). RNA sequencing libraries were prepared using a TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA). Double-stranded cDNA libraries were quantified, quality checked, normalized, and pooled according to their concentrations. Pooled libraries were run on a 2% agarose gel to size select fragments of ~240–600 bp. DNA was recovered using a Geneclean III kit (MP Biomedical, Santa Ana, CA) and purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). Sequencing was conducted at the UCI Genomics High-Throughput Facility using a HiSeq 2500 (Illumina, San Diego, CA), paired end 100-cycle sequence run. Biological replicate numbers for females (F) and males (M) for each species were as follow: *H. charithonia* (F = 6, M = 6), *H. sara* (F = 5, M = 6), *H. erato* (F = 3, M = 3), *H. doris* (F = 6, M = 6), *H. melpomene* (F = 4, M = 4).

Transcriptome De Novo Assembly

RNA-Seq reads were trimmed using TQSfastq_gz.py script (version 1.5) written by Rene L. Warren as published by the Free Software Foundation. Reads were end-trimmed removing nucleotides with a Phred score <20 and keeping all the reads longer than 30 bp. After trimming unpaired reads were discarded using a perl script kindly provided by Peter

Andolfatto, Princeton University. For *H. charithonia*, *H. sara*, and *H. doris de novo* Trinity (version 2.1.1) transcriptome assembly, one female and one male library were used per species, by selecting the libraries containing the highest number of reads. For *H. melpomene*, RNA-Seq data from antennae, mouthparts, and legs of one male and one female were obtained from ArrayExpress: E-MTAB-6810 (supplementary table S25, Supplementary Material online; Briscoe et al. 2013). A *de novo* assembly was made using these six libraries in addition to a male and female head RNA-Seq library. For *H. erato*, two specimens with male-like *UVRh1* expression were used for the transcriptome assembly. To ensure that the *H. erato* transcriptome was not male biased, a second transcriptome was assembled using different individual specimens, one female and one male with female- and male-like *UVRh1* expression, respectively, and all the analyses reported here were redone using the second transcriptome (supplementary figs. S8–S12, Supplementary Material online). Because the assembly and annotation quality of the first assembled *H. erato* transcriptome was higher and the results obtained from both transcriptomes were highly congruent, we decided to present the results of the first transcriptome. A contig for *UVRh1* failed to be assembled in both of the *H. erato* transcriptomes, so count and FKPM values for *UVRh1* were obtained by mapping the whole transcriptome reads to the complete coding sequence of *UVRh1* (accession number: AY918904.1, Zaccardi et al. 2006) Count and FPKM values for Kr-h1 were obtained from transcriptome 2.

Transcriptomes were assembled allowing for a minimum of 300 bp contig length (Haas et al. 2013). The *de novo* Trinity assemblies had from 31,193 to 40,275 assembled genes with N50 ranging from 2,395 to 3,310 bp (see supplementary table S1, Supplementary Material online, for a summary of assembly statistics). Reads were mapped back to the transcriptome assembly using Bowtie (version 1.0.0), and raw read counts were estimated with RSEM (version 1.3.0) as implemented in the Trinity pipeline (Langmead et al. 2009; Li and Dewey 2011) (see supplementary table S2, Supplementary Material online, for mapping statistics). Correlation between biological replicates for each species and within each sex was tested using a Spearman's correlation test, where a significant correlation within samples was found, with correlation coefficients ranging from 0.86 to 0.93 (supplementary table S3, Supplementary Material online).

Identification of Sex-Biased Genes

Sex-biased genes were identified using the Bioconductor package edgeR (3.13.4) as implemented in R (version 3.0) (Gentleman et al. 2004; Robinson et al. 2009; R Core Team 2015). A gene-specific, tagwise dispersion was used to explain experimental variation and a generalized linear model was fitted to a one-factor analysis. Calculated *P* values were corrected for multiple testing using the Benjamini–Hochberg adjustment (Benjamini and Hochberg 1995). Sex-biased genes were classified as those genes showing a false discovery rate (FDR) < 0.05. To visualize the distribution of all expressed genes, including the identified sex-biased genes, in relation to the average count per million and the male/female fold differential expression, we plotted MA plots for the five species.

Transcriptome Annotation

To identify candidate coding regions from the *de novo* Trinity transcriptome assemblies, we used TransDecoder (version 5.0.2), which identifies likely coding sequences (Haas et al. 2013). The longest contig for each Trinity transcriptome component was used for open reading frame prediction. TransDecoder identified ORFs that were at least 100 amino acids long. Whole transcriptomes from *H. charithonia*, *H. sara*, *H. erato*, *H. doris*, and *H. melpomene* were annotated by identifying orthologous hits in UniProt, Flybase, and Pfam databases using blastp (2.2.30) and keeping only hits with an *e*-value $< 10^{-3}$ (Altschul et al. 1990; Chintapalli et al. 2007; Punta et al. 2012) (Dryad data identifier: DOI: doi: 10.5061/dryad.ds21fv5). For those Trinity genes having more than one coding sequence predicted, the coding sequence with the lowest *e*-value was kept. To identify whole transcriptome and shared sex-biased genes among species with shared annotation hits, we built a five-way Venn diagram using the "VennDiagram" package (version 1.6.18) as implemented in R. A deviation of the expected shared sex-biased genes across the species was tested by a Fisher's exact test.

Orthology Assessment

Orthologous protein sequences from *H. charithonia*, *H. sara*, *H. erato*, *H. doris*, and *H. melpomene* were identified as described in (Ballesteros and Hormiga 2016) using their Unrooted Phylogenetic Orthology (UPhO) (version 1.0.0) method. Firstly, we performed an all species pairwise blastp search to start defining sequence similarity. To refine the sequence relationships found with blastp, we used a Markov clustering algorithm (MCL) (version 1.0.0) (Enright et al. 2002) that uses blastp *e*-values and sequence length to define protein clusters. The inflation value in the MCL algorithm defines how "loose" or "tight" the defined clusters are. Here, we investigated inflation values ranging from 1.5 to 7, with an inflation value of 2, resulting in the highest number of sequence clusters. Fasta files obtaining the clustered protein sequences were generated and aligned with MAFFT (version 7.305) (Katoh and Toh 2008). Gappy regions were removed using trimAl (version 1.3) (Capella-Gutiérrez et al. 2009) and sequences of at least 50 bp length were kept for further analysis. Phylogenetic inference of the orthoclusters was done using RAxML (version 8.2.10) (Stamatakis 2006), with 100 rapid bootstraps with automatic protein model selection plus gamma (-m PROTGAMMAAUTO). Orthology was then assessed from each generated tree using the UPhO algorithm (UPhO.py), which uses a species overlapping algorithm as described by (Gabaldón 2008), without the requirement of a rooted tree which could biased orthology inference (Ballesteros and Hormiga 2016). Sex-biased genes were identified in the set of orthologous genes and a deviation of the expected number of shared sex-biased genes among the five species was compared with transcriptome-wide expectations and tested with a Fisher's exact test.

Assessment of Directional Selection on Gene Expression in Sex-Biased Genes

The Δx statistic was calculated to assess directional selection on gene expression in the sex-biased genes identified. The statistic was implemented as described before (Moghadam et al. 2012; Dean et al. 2015), using *H. melpomene* as the reference species for the calculation of expression divergence. When Δx was calculated for *H. melpomene*, *H. doris* was used as the reference species. For the most common sample size (n), the median for all sample sizes was used, n = 11. To identify orthologous sex-biased genes between the reference and the focal species, reciprocal blastn was performed and the transcript with the lowest *e*-value was selected for the analysis. FPKM values were $\log_2$ transformed.

Evolutionary Rates Analysis (d_N/d_S)

Sex-biased and unbiased predicted coding sequences from *H. charithonia*, *H. sara*, *H. erato*, and *H. doris* were reciprocally blasted against the latest *H. melpomene* whole genome coding sequences, filtering out hits with *e*-value $>10^{-3}$ (Davey et al. 2016). To estimate d_N/d_S in *H. melpomene*, the *H. doris de novo* assembly was used to blast sex-biased and unbiased genes found in *H. melpomene*. For the d_N/d_S estimates of unbiased genes, $\sim$ 500 unbiased genes were randomly selected. TranslatorX (version 1.0.0) was used to translate and back-translate sequence pairs that were aligned using MUSCLE (version 3.8), thus ensuring codon-to-codon alignment (Edgar 2004; Abascal et al. 2010). A portion of the alignments were checked manually using AliView (version 1.18.1) (Larsson 2014) and all alignments having nucleotides mismatches >45% were filtered out for downstream analyses. This selects for sequence similarity, making our d_N/d_S estimates more conservative. To test for protein substitution rates (d_N/d_S), synonymous substitution (d_N) and nonsynonymous (d_S) rates in sex-biased and unbiased genes were estimated using CodeML as implemented in PAML (4.1) (runmode=−2, CondonFreq = 3). To test if female-, male-, all sex-biased or unbiased genes have an effect on d_N/d_S rates we fitted the following generalized linear model: glm ($d_N/d_S \sim$ expression type, family="Gamma"). We used the R package "fitdistrplus" (version 1.0.9) to help us identity which distribution would explain our d_N/d_S rates best and used goodness-of-fit statistics (Kolmogorov–Smirnov and Cramer–von Mises) to test how well our data is described by the distribution proposed. Additionally, differences in d_N and d_S between sex- and unbiased genes were assessed using a Wilcoxon test and 95% confidence intervals of their median estimates were calculated using R (R Core Team 2015). To investigate codon usage bias, we used ENCprime (version 1.08.0) (Novembre 2002), which implements the Nc statistic, which describes the effective number of codons used in a gene as a measure of the strength of departure of equal synonymous codon usage in a gene (Wright 1990). A Wilcoxon test was applied to detect a deviation of the distribution of codon usage between sex-biased and unbiased genes.

Dosage Compensation

FPKM (Fragments Per Kilobase of transcript per Million mapped reads) matrices were generated using the Trinity suite script abundance_estimates_to_matrix.pl script. FPKM values were normalized across samples using a TMM normalization as described in (Robinson and Oshlack 2010) and implemented in Trinity (Haas et al. 2013). Chromosomal identity for four species (*H. charithonia*, *H. sara*, *H. erato*, and *H. doris*) was assessed by blast (version 2.2.30) (Altschul et al. 1990), using our *de novo* Trinity transcriptome assemblies for each species as the query against the *H. melpomene* v2.0 genome (Davey et al. 2016). For each contig the best hit was kept and hits with an *e*-value $> 10^{-3}$ were discarded. The bias that can be introduced by an overrepresentation of non- or very low expressed genes in a sample when assessing dosage compensation patterns has been intensely discussed (Xiong et al. 2010; Deng et al. 2011). To avoid confounding signals in our dosage compensation analysis, we explore two different filtering strategies. In the first one, genes with a mean expression lower than FPKM < 1, FPKM < 3, and FPKM < 5 were filtered out of the analysis. The mean expression for each gene was drawn by averaging its expression in all replicates. Our second filtering approach was to identify a minimum expression threshold by characterizing the observed $\log_2$ FPKM expression distributions by using a Kolmorogov–Smirnov statistic and multivariate adaptive regression splines as descried in the data-adaptive flag method (DAFS) (George and Chang 2014). For the comparison of autosomal and Z-chromosome expression within each sex, we used the mean expression of all the replicates for each gene, within each sex. The gene expression within replicates for each sex for each species was significantly correlated in all species, which we tested using a Spearman's rank order correlation test (supplementary table S3, Supplementary Material online). Deviation of the female: male expression ratio on the autosomes and the Z-chromosome was tested using a two-sample Wilcoxon test. A Bonferroni correction was additionally done to correct for type I error due to multiple testing. The Z:A expression ratio for females and males was calculated by dividing the median of all expressed genes in the Z-chromosome by the median of all genes expressed on the autosomes. To calculate confidence intervals for Z:A expression ratios, we performed random sampling with replacement for the Z and autosomal expressed genes for each sex and species. The ratios of 10,000 replicates were used to calculate the 95% CI. All analyses were performed in R.

Chromosomal Enrichment Analysis

To identify chromosomal locations of sex-biased and unbiased genes in *H. charithonia*, *H. sara*, *H. erato*, and *H. doris* a nucleotide blast search was done, using the *de novo* assembled transcriptomes against the latest *H. melpomene* genome (Davey et al. 2016). Reciprocal BLAST hits with *e*-value $>10^{-3}$ were removed from the analysis. Inferring chromosomal location within *Heliconius* this way is a reasonable approach since high conservation of chromosomal synteny has been reported within Lepidoptera (Pringle et al. 2007; Dasmahapatra et al. 2012). Chromosomal counts for sex-

biased and unbiased genes were calculated and a 2×2 contingency table was built in order to perform a Fisher's exact test to test for a deviation in the proportion of sex-biased and unbiased genes located in the Z-chromosome versus the autosomes.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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