1 **Title**

2

3 **Drift and directional selection are the evolutionary forces driving gene expression**

4 **divergence in eye and brain tissue of *Heliconius* butterflies.**

5

6 Ana Catalán[1,3], Adriana Briscoe[2], Sebastian Höhna[3,4].

7 [1] Department of Evolutionary Biology, Evolutionary Biology Centre (EBC), Uppsala

8 University, Norbyvägen 14-18 75236, Uppsala, Sweden.

9 [2] Department of Ecology and Evolutionary Biology, University of California, Irvine, CA

10 92697, USA.

11 [3] Division of Evolutionary Biology, Ludwig-Maximilians-Universität München,

12 Grosshaderner Straße 2, Planegg-Martinsried 82152, Germany.

13 [4] GeoBio-Center, Ludwig-Maximilians-Universität München, Richard-Wagner Str. 10,

14 80333 Munich, Germany

15

16 Corresponding author: ana.catalan@gmail.com

17

18 **Key words:** Brownian motion, natural selection, stabilizing selection, Ornstein-

19 Uhlenbeck, RevBayes.

20

21

22

23

Abstract

Investigating gene expression evolution over micro- and macroevolutionary timescales will expand our understanding of the role of gene expression in adaptation and speciation. In this study, we characterized which evolutionary forces are acting on gene expression levels in eye and brain tissue of five *Heliconius* butterflies with divergence times of ~5-12 MYA. We developed and applied Brownian motion and Ornstein-Uhlenbeck models to identify genes whose expression levels are evolving through drift, stabilizing selection, or a lineage-specific shift. We find that 81% of the genes evolve under genetic drift. When testing for branch-specific shifts in gene expression, we detected 368 (16%) shift events. Genes showing a shift towards up-regulation have significantly lower gene expression variance than those genes showing a shift leading towards down-regulation. We hypothesize that directional selection is acting in shifts causing up-regulation, since transcription is costly. We further uncover through simulations that parameter estimation of Ornstein-Uhlenbeck models is biased when using small phylogenies and only becomes reliable with phylogenies having at least 50 taxa. Therefore, we developed a new statistical test based on Brownian motion to identify highly conserved genes (i.e., evolving under strong stabilizing selection), which comprised 3% of the orthoclusters. In conclusion, we found that drift is the dominant evolutionary force driving gene expression evolution in eye and brain tissue in *Heliconius*. Nevertheless, the higher proportion of genes evolving under directional than under stabilizing selection might reflect species-specific selective pressures on vision and brain necessary to fulfill species-specific requirements.

Introduction

Species and populations diverge through the accumulation of genetic changes that affect coding or non-coding genomic regions. Genetic variation affecting gene expression has the potential of changing gene expression patterns in a spatiotemporal manner, by changing gene expression profiles in specific organs and cell types at particular developmental stages (Carroll 2005; Signor and Nuzhdin 2018). This spatiotemporal attribute of gene expression might enable evolutionary change in a compartmentalized way, allowing for change where it is required but also allowing for the needed processes to remain conserved. Phenotypic diversity caused by changes in gene expression encompasses a great variety of traits, including changes affecting an organism's coloration (Nadeau 2016), size and shape (Ahi et al. 2017), as well as sensory perception and behavior, amongst other phenotypes (Lee et al. 2000; Wanner et al. 2007). Even though major advances have been made in linking gene expression variation to a phenotype (Catalán et al. 2016; Glaser-Schmitt and Parsch 2018), discerning the evolutionary forces shaping gene expression level variation among closely related species is an area that needs further research.

To understand the evolutionary forces acting on gene expression it is necessary to model within and between species gene expression variance. Neutral gene expression divergence between species leads to gene expression difference through divergence alone. Thus, neutral changes in gene expression modeled by random drift provides a null hypothesis to detect deviations from the expected neutral gene expression divergence. A linear relationship between divergence time and gene expression variance difference has been proposed for closely related species, assuming a clock-like (i.e., constant through

time) rate of gene expression divergence (Khaitovich et al. 2004; Khaitovich 2005). Another approach to studying the evolutionary forces shaping gene expression evolution, which is motivated by statistical phylogenetics, is fitting Brownian motion (BM) models. BM-models are often used to describe changes in continuous trait through time using random drift with rate σ^2 and taking into account the known phylogeny of the taxa of interest (Felsenstein 1985). Thus, in a BM context, σ^2 can also be described as the volatility parameter that determines the rate at which a trait's value diffuses away from its current state (Bedford and Hartl 2009). Fitting BM models to investigate gene expression evolution has shown that stabilizing selection and evolution through genetic drift can be readily characterized (Kalinka et al. 2010; Wong et al. 2015).

Ornstein-Uhlenbeck (OU) models have also been used to study continuous trait evolution in a phylogenetic context (Hansen 1997; Butler and King 2004). Ornstein-Uhlenbeck models, an extension to BM-models, include two extra parameters, α and θ, for modeling the strength of stabilizing (α) selection towards a phenotypic optimum (θ). As in a BM context, σ^2 is the rate at which a trait changes through time, and α is the force pulling back the diffused trait to an optimum state. This is analogous to stabilizing selection pulling back a trait to its optimum value after having experienced a departure from it. Theta (θ) is described as the trait's optimum state at a particular time point toward which the pull of α is aimed (Hansen 1997; Butler and King 2004). OU-models offer a useful framework to generate hypothesis about the evolutionary forces acting on transcriptome levels, whether it is drift, stabilizing selection or directional selection (Bedford and Hartl 2009; Rohlfs and Nielsen 2015; Wong et al. 2015; Chen et al. 2017; Stern and Crandall 2018).

In this study, we used five closely-related species of *Heliconius* butterflies to explore the evolutionary forces shaping gene expression variation in eye and brain tissue. *Heliconius charithonia*, *H. sara*, *H. erato*, *H. melpomene* and *H. doris* (Figure 1) belong to four of the seven distinct *Heliconius* phylogenetic clades with divergence times ranging from 5.5 to 11.8 MYA. Beside showing a great diversity in wing color patterns (Kronforst and Papa 2015), *Heliconius* butterflies also show a diversity of life history traits (Salcedo 2010; Merrill et al. 2015), mating systems (Beltrán et al. 2007; Walters et al. 2012) and behavior (Mendoza-Cuenca and Macías-Ordóñez 2005). Since *Heliconius* butterflies are diurnal species, visual stimuli provide key sources of information about the environment. For example, flowers and oviposition sites, potential mates or predators are all targets of interest to butterflies in which the first line of perception is visual (Finkbeiner et al. 2014; Finkbeiner et al. 2017). After visual cues are detected by the visual system, the detected information travels to the brain, where it is processed and its output can result in a specific behavior or physiological response. Thus, the brain's processing and output together with the visual system have the potential of being finely tuned according to a species' life history. In the case of *Heliconius* butterflies, a high diversity of adult compound eye retinal mosaics (between sexes and species) has been discovered (McCulloch et al. 2017), as well as species-specific differences in brain morphology (Montgomery et al. 2016). Which evolutionary forces are shaping adult eye and brain expression in *Heliconius* is one question we seek to investigate, and in that way, gain an understanding into the potential role of inter-species gene expression differences in speciation and adaptation.

115 Therefore, in this study we investigated which evolutionary forces are driving
116 gene expression variation in eye and brain tissue. More specifically, we aimed to identify
117 if expression variation in individual genes is evolving, for example, through drift,
118 stabilizing selection or directional selection. To this end, we generated a set of
119 orthoclusters shared among our five butterfly species together with expression data for
120 each gene in each orthocluster. We characterized the selective forces acting on gene
121 expression levels thereby revealing the fraction of the transcriptome evolving under drift,
122 directional selection, as well stabilizing selection.
123
124 **Methods**
125 *Data set*
126 The data set used in this study was published in (Catalán et al. 2018) and retrieved
127 from ArrayExpress: E-MTAB-6810 and Dryad data identifier: DOI: doi: 10.5061/
128 dryad.ds21fv5. In Catalán et al (2018), whole transcriptomes were generated from eye
129 and brain tissue combined for *Heliconius charitonia*, *H. sara*, *H. erato*, *H. doris* and *H.*
130 *melpomene*. *De novo* transcriptome assemblies were generated for each species and the
131 corresponding reads were mapped back to their matching transcriptome using Bowtie
132 (version 1.0.0). Raw read counts and FPKM values were calculated for each species and
133 used for downstream analysis. TransDecoder (version 5.0.2) was used to identify
134 candidate coding regions from each *de novo* Trinity transcriptome. The predicted coding
135 sequences were utilized to annotate each transcriptome by identifying orthologous hits in
136 UniProt, Flybase and Pfam databases using blastp (2.2.30) and keeping only hits with an
137 *e*-value < 10^{-3} (Altschul et al. 1990; Chintapalli et al. 2007; Punta et al. 2012).

Orthology assessment

The set of orthoclusters used to assess gene expression evolution across *Heliconius* was retrieved from supplementary table S15 published in Catalán *et. al.* 2018. Briefly, the Unrooted Phylogenetic Orthology (UPhO) pipeline and model was used to assess orthologous relationships between the five *Heliconius* species (Ballesteros and Hormiga 2016). UPhO uses an all species pairwise blastp search and a Markov clustering algorithm (MCL) (version 1.0.0) (Enright et al. 2002) to cluster sequences according to sequence similarity. Clustered sequences were aligned with MAFFT (version 7.3.05) (Katoh and Toh 2008) and curated after alignment with trimAl (version 1.3) (Capella-Gutiérrez et al. 2009). Phylogenetic inference for each sequence cluster was done using RAxML (version 8.2.10) (Stamatakis 2006) and orthology was assessed for each generated tree using the UPhO algorithm. A matrix with $\log_2$ FPKM values was generated for each orthoclusters which was used to analyze gene expression variance.

Modelling Gene Expression Evolution

Table 1. Summary of the models implemented in this work

Model	Description	Parameters
Equal species means	All species have the same mean gene expression level.	μ – global mean gene expression level
Unequal species means	All species have their own independent mean gene expression level.	μ_i – mean gene expression level per species
Brownian motion (BM)	Random drift of the species mean gene expression level along the phylogeny.	σ^2 - rate of drift
Brownian motion (BM) with shift	Random drift with one branch having a different rate (directional selection).	σ^2_B - rate of drift background branch

		σ^2_F - rate of drift foreground branch
Ornstein-Uhlenbeck (OU)	Stabilizing selection of the species mean gene expression level evolving along the phylogeny.	σ^2 - rate of drift α - strength of selection θ - optimal gene expression level
Ornstein-Uhlenbeck (OU) with shift	Directional selection due to a shift in optimal gene expression level.	σ^2_B - rate of drift background branch σ^2_F - rate of drift foreground branch. θ_B - optimal gene expression level background branch θ_F - optimal gene expression level foreground branch α - strength of selection

To study the forces driving gene expression evolution, we implemented a set of six different statistical models (Table 1). Each model models the mean species gene expression level (between-species variance) and the gene expression levels of individual samples per species (within-species variance). How these mean species gene expression levels evolve, or not, along the phylogeny and over time, is specific and central to each model. We estimated the parameters of each model and performed Bayesian model selection using Bayes factors to establish which model describes the observed data best and thus which process is most likely to drive gene expression evolution in the five *Heliconius* species of our study (see below).

The simplest model of gene expression assumes that all species have the exact same mean gene expression level. In this case, we only have one parameter μ which defines the mean gene expression level of all species. The expression level X_{ij} of individual *i* from species *j* is modeled using a normal distribution with $X_{ij} \sim \text{Norm}(\mu, \delta^2_i)$.

We chose a uniform prior distribution between -20 and 20 for the mean gene expression parameter μ. Note that we assume that every species has its own gene expression variance parameter δ^2_i (see below). This model assumes there is no evolution of gene expression levels, i.e., gene expression levels are completely conserved among species.

The second model that we implemented was a model where each species has its own gene expression mean μ_i. Thus, we model the gene expression level X_{ij} of gene *i* from species *j* using a normal distribution with $X_{ij} \sim \text{Norm}(\mu_i, \delta^2_i)$. In this model, each species has a different mean gene expression level, but these gene expression levels do not evolve under an evolutionary model; they are intrinsically different without any mechanistic reason (no phylogenetic signal). As with the first model, we assumed a uniform prior distribution between -20 and 20 for each mean gene expression level μ_i.

The third model that we implemented was a phylogenetic Brownian motion model (Felsenstein 1985). We assume that any gene expression value at the root of the phylogeny is equally probable. Then, the mean gene expression levels μ evolve along the lineages of the phylogeny. The Brownian motion model specifies that the focal variable, μ in our case, is drawn from a normal distribution centered around the value of the ancestor, μ_A. The amount of change, i.e., rate of random drift, is defined by the parameter σ^2. We assumed a log-uniform prior distribution between 10E-5 and 10E5 for the drift parameter σ^2. Thus, the mean gene expression levels μ_i for the species of the phylogeny are distributed according to a multivariate normal distribution where the covariance structure is defined by the phylogeny (Felsenstein 1985). This means that more closely-related species are expected to have a more similar mean gene expression level because they share more evolutionary history (i.e., they are more recently diverged), which is

192 modeled by the covariance structure. Such a phylogenetic model of gene expression
193 evolution has been applied previously by (Bedford and Hartl 2009). Importantly, the BM
194 model only defines how the mean gene expression levels evolve but does not allow for
195 any sample variance of the individuals of a species. Therefore, we extended the standard
196 phylogenetic BM model to allow for within-species sample variance where again the
197 expression level X_{ij} of individual *i* from species *j* is normally distributed with $X_{ij} \sim$
198 $\text{Norm}(\mu_i, \delta^2_i)$ where δ^2_i is the within-species variance parameter (Ives et al. 2007; Rohlfs
199 and Nielsen 2015). This extension to allow for within-species variance was developed for
200 all phylogenetic models (BM, BM with shift, OU and OU with shift).
201 The fourth model that we implemented was a phylogenetic BM model with
202 branch-specific rates of evolution, thus detecting directional selection. The mean gene
203 expression level evolves under a BM model (i.e., random drift) where the rate of
204 evolution for branch *k* is given by σ^2_k (O'Meara et al. 2006; Eastman et al. 2011). Thus,
205 a branch with a higher rate of evolution σ^2_k signifies more change in gene expression
206 levels than under a constant rate random drift model (the BM model). Directional
207 selection can therefore be detected by inferring an elevated estimate of σ^2_k compared
208 with the background rate of drift σ^2. Specifically, we applied a background rate of drift
209 σ^2_B to all branches except the chosen foreground branch which received its own rate of
210 drift σ^2_F.
211 The fifth model we implemented was a phylogenetic Ornstein-Uhlenbeck process
212 (Hansen 1997). The Ornstein-Uhlenbeck (OU) process models, similar to BM, the
213 evolution of the mean gene expression level per species along a phylogenetic tree.
214 However, unlike BM, the mean expression level diffuses with rate σ^2 and is attracted

with strength α to an optimum level θ. Thus, the OU process has an expected variance of $\sigma^2/2\alpha$ which is independent of time, i.e., does not increase with increasing time but instead stabilizes (c.f. Figure 10). The variance becomes small if either the strength of selection is large or the rate of drift is small. This is, in fact, a major problem for OU models which cannot distinguish if attraction (or selection) is strong or diffusion is weak (Ho and Ané 2014; Cooper et al. 2016).

The sixth model we implemented was an Ornstein-Uhlenbeck process with a branch-specific shift in both the rate of drift σ^2 and the optimum gene expression level θ (Rohlfs et al. 2014; Uyeda and Harmon 2014). Thus, this branch-specific OU model is analogous to the branch-specific BM model, allowing for directional selection in an OU framework. Specifically, we tested if there was a significant support for the chosen foreground branch which received its own rate of drift σ^2_F and optimum θ_B to be different from the background rate of drift σ^2_B and optimum θ_B. We used the same prior distributions as before and assumed that both parameters for the background and foreground branches are drawn from the identical prior distribution. This model has in total five free additional parameters along with the five nuisance parameters (the within-species variances). Thus, we expect that this model is more prone to be over-parameterized for our dataset with five species. Nevertheless, our Bayesian approach for parameter estimation and model selection integrates over parameter uncertainty and penalizes extra parameters by integrating over the prior distribution.

238 *Parameter Estimation and Model Selection*

239 We estimated parameters for our different models in a Bayesian statistical
240 framework. Thus, we approximated the posterior distribution of the model parameters
241 using Markov chain Monte Carlo sampling (Metropolis et al. 1953; Hastings 1970). We
242 ran a separate MCMC analysis for each model and gene, 2393 analysis per model. Every
243 model parameter was updated twice per MCMC iteration where the order of parameter
244 updates was chosen randomly. We applied the same settings of the MCMC algorithm for
245 each model. First, a burn-in phase of the MCMC algorithm was run for 2,000 iterations
246 with auto-tuning every 100 iterations. Then, the actual MCMC simulation was run for
247 50,000 iterations with sampling 10 iterations, yielding 5,000 samples from the posterior
248 distribution (Höhna et al. 2017).

249 Model selection was performed using marginal likelihoods. Marginal likelihoods
250 are the probability of the data for a specific model integrated over all possible parameter
251 values. From the marginal likelihood we can then compute Bayes factors and model
252 probabilities (i.e., weights of a model being the true model generating the data given a set
253 of candidate models). We approximated the marginal likelihoods using stepping stone
254 sampling (Fan et al. 2011). The stepping stone algorithm implemented in RevBayes
255 consisted of 128 MCMC runs where each MCMC ran had the likelihood function raised
256 to the power of β computed by the quantiles of a beta probability distribution (Höhna et
257 al. 2017).

258

259

260

Data availability

The five different models that we used in our study are implemented in Bayesian phylogenetic inference software RevBayes v1.0.8 (Höhna et al. 2016). For efficient computations, we implemented the restricted maximum likelihood (REML) algorithm for BM models (Felsenstein 1985) and OU models (Fitzjohn 2012; Freckleton 2012). The source code and compiled executables of RevBayes are available from https://github.com/revbayes/revbayes and tutorials about the analyses are available from https://revbayes.github.io/tutorials/.

Results

To assess the evolutionary forces acting on gene expression levels in *H. charithonia*, *H. sara*, *H. erato*, *H. doris* and *H. melpomene* (Figure 1), we used the gene orthology dataset, composed of 2373 orthologous genes, published before (Catalán et al. 2018). From this previous work, we also obtained FPKM (Fragments per Kilobase per Million mapped read) values for each gene and sample. The $\log_2$ transformed FPKM values were used to build an expression matrix and to model gene expression evolution.

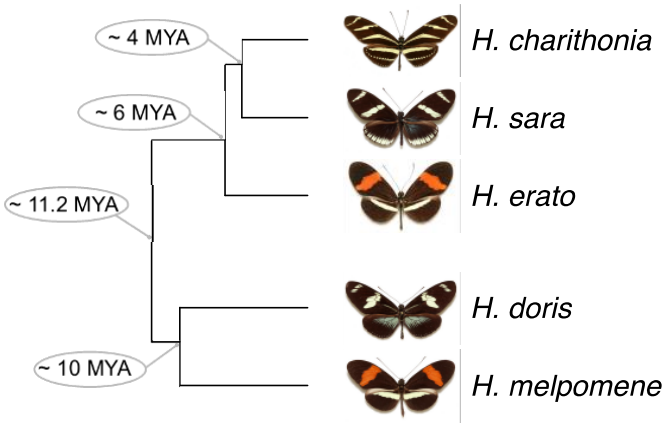


Figure 1 Phylogenetic relationship of the *Heliconius* species used in this study showing divergence times at each node (Kozak et al. 2015).

Testing for equality of within-species variance in gene expression levels

Equality of variances among populations or samples drawn from a normal distribution is usually assumed when testing for differences in mean values obtain from continuous data or gene expression data as in our case (Warnefors and Eyre-Walker 2012; Rohlfs et al. 2014). Assuming equality of variances when it is not the case can lead to high Type I error rates (Gastwirth et al. 2009). To avoid assuming equality of variances, we computed the within-species variances for each orthocluster and checked for the presence of a correlation across species. From a pairwise assessment of within-species variance we found no significant correlation among all possible pairs, with Pearsons's rho values ranging from 0.07 to 0.2 (Figure 2). Since gene expression variance across species is heterogeneous, hence not correlated among species, we treated within-species variance as a random variable when fitting BM and OU models.

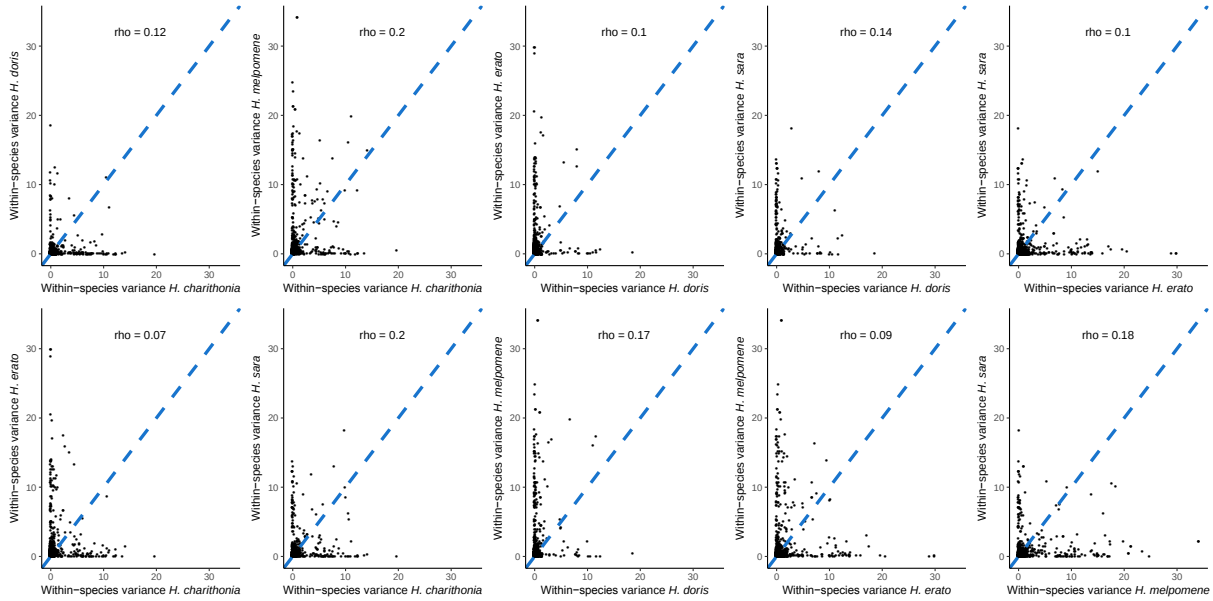


Figure 2 Pairwise correlation between five *Heliconius* species and their gene expression within-species variance. The correlation strength between within-species variances was estimated by calculating Pearson's correlation coefficient, rho ranging from 0.07 to 0.2.

Testing for gene expression evolution through drift using Brownian motion models

We applied BM models to describe changes in gene expression levels through random drift. As the alternative hypotheses, we used two non-phylogenetic models where either all species had identical mean gene expression levels (Model 1) or all species had their own independent mean gene expression levels (Model 2). For each gene we computed the probability of the BM model having produced the observed data, i.e., a high probability means that it is more probable that the gene expression levels evolved under a BM model whereas a low probability means that it is more probable that the gene expression levels evolved under a non-phylogenetic model (Model 1 and Model 2). A model probability of >0.75 corresponds to a Bayes factor of >3 (positive support) and a model probability of >0.95 corresponds to a Bayes factor of >20 (strong support).

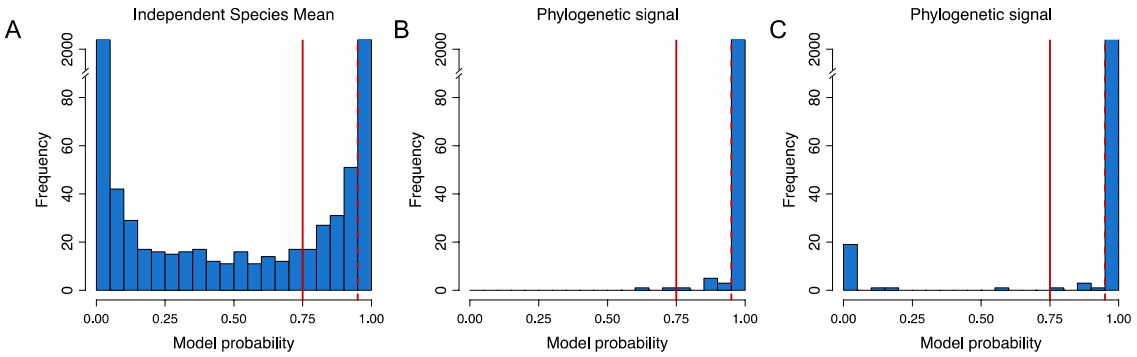


Figure 3. Testing for random drift in gene expression levels of *Heliconius* using Brownian motion. Significance is shown at model probability > 0.75 (solid red, Bayes factor > 3, positive support) and model probability > 0.95 (solid red, Bayes factor > 20, strong support). (A) Shows the comparison between the two non-phylogenetic models (identical vs independent species mean). (B) Shows the model probability of the BM model compared with the independent species mean model. (C) Shows the model probability of the BM model compared with the identical species mean model.

Our results show that the majority of gene expression levels (2369 out of 2393) are evolving as expected given the known *Heliconius* species phylogeny, i.e., that there is a strong phylogenetic signal (Figure 3). These results indicate that drift is the dominating evolutionary force driving transcriptome change. Only a small fraction of the orthoclusters did not show a phylogenetic signal, opening the question of the putative genetic forces shaping gene expression levels of these genes.

331 *Testing for conserved gene expression level*

332 The next question we explored was how prevalent conserved gene expression
333 levels are in eye and brain tissue of *Heliconius*. This question could be answered with our
334 previous results by computing how often Model 1, with identical species means, was
335 recovered (Figure 2C). However, our model selection procedure relied on computing
336 marginal likelihoods which are intrinsically sensitive to the choice of prior distribution
337 (Berger 1990; Kass and Raftery 1995; Sinharay and Stern 2002). Therefore, we
338 additionally performed a sensitivity analysis of $\sigma^2 = 0$ using Monte Carlo simulation as
339 follows (Goldman 1993). We estimated the posterior distribution of all parameters under
340 the identical species mean model (the only parameters were the within-species variances),
341 then we used 1,000 parameter samples from the posterior distribution to simulate gene
342 expression datasets (e.g., a dataset consisting of a single gene with five species and 6-12
343 individuals per species) under the identical species mean model, yielding 1,000 simulated
344 datasets per gene in total. Then, for every gene of the 2393 genes, we estimated σ^2 for
345 each simulated dataset as well as the original dataset, which amounted to a total of
346 2,395,393 MCMC analyses. Finally, we calculated if the mean posterior estimate of the
347 empirical dataset was larger than 95% of the mean posterior estimates of the simulated
348 datasets. In the cases when the mean posterior estimate of σ^2 was not larger than the
349 mean estimate of 95% of the simulated datasets we concluded that these genes are highly
350 conserved (Figure 4).
351

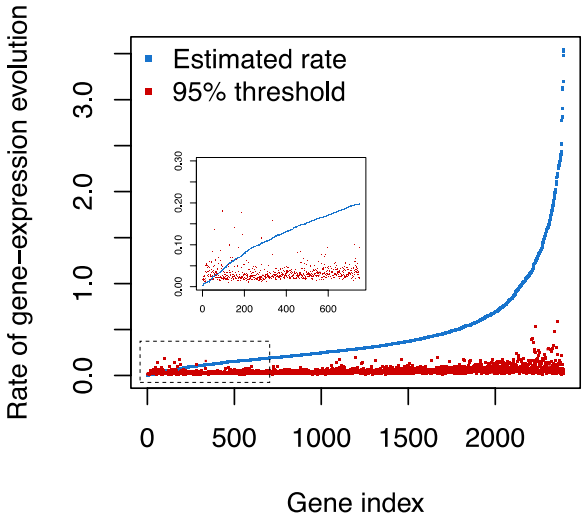


Figure 4. Posterior mean estimates of the drift rate σ^2 (blue) and the 95% threshold computed (red) using Monte Carlo simulations. The genes were sorted by an ascending estimate of σ^2. Inset: close-up of genes whose σ^2 is not significantly bigger than zero.

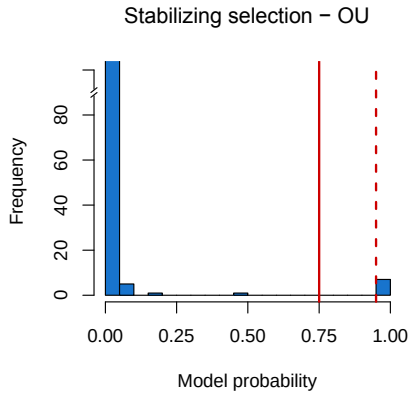
By using the described approach, we uncover a set of 83 orthoclusters whose gene expression variance across species is highly conserved (Figure 4 and Figure S1). A sigma squared not significantly different from zero, can be caused by stabilizing selection hindering gene expression divergence, resulting in more similar gene expression patterns across different *Heliconius* species.

Testing for stabilizing selection acting on gene expression levels

Subsequently, we moved forward into implementing an Ornstein-Uhlenbeck model (OU) to investigate the strength of stabilizing selection (Bedford and Hartl 2009; Rohlfs et al. 2014). OU-models are an extension of BM-models, in which they include two extra parameters, α and θ. In a BM context, if σ^2 is the rate at which a trait changes

368 through time, α is then described as a force pulling back the diffused trait to an optimum
369 state (θ).

370 We estimated the marginal likelihood for each gene under a BM model and an
371 OU model. Then, we computed the probability (i.e., support) of an OU model over a BM
372 model using the marginal likelihoods. Our results show a very low support for stabilizing
373 selection (Figure 5). When the marginal likelihoods were examined, in 99.7% of the
374 cases a BM model explained our expression data better than an OU-model.



375

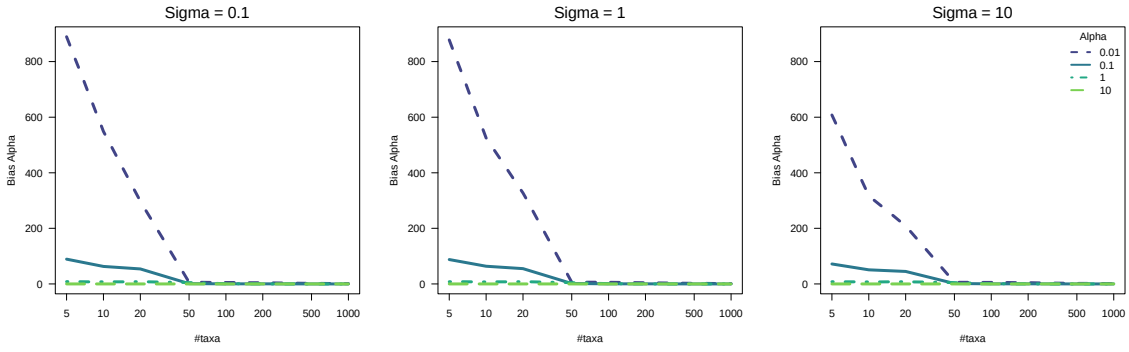
376 **Figure 5.** Model probability when testing the strength of alpha when fitting an OU model
377 for the assessment of stabilizing selection. Significance is shown at model probability >
378 0.75 (solid red, Bayes factor > 3, positive support) and model probability > 0.95 (solid red,
379 Bayes factor > 20, strong support). There are only 7 genes with a significant support for
380 stabilizing selection.

381

382 *Testing the power to estimate stabilizing selection*

383 Our results indicating that very few genes evolved under stabilizing selection
384 conflict with previous findings (Bedford and Hartl 2009). However, it has previously
385 been discussed that when working with small phylogenies (less than ten species) there is

386 a lack of power for parameter estimation when using an OU-model (Rohlfs et al. 2014).
387 By simulating data under an OU model using phylogenies with varying numbers of taxa,
388 we were able to show how parameter estimation is biased. The attraction parameter α
389 could only be estimated closely to the true values used for the simulations when the
390 phylogenies contained 50 or more taxa. Thus, we can observe that the bias observed for
391 parameter estimation drops considerably when the number of taxa composing the
392 phylogeny reaches 50 (Figure 6). This observation holds as well for the estimation of σ_2
393 under a range of sigma values (Figure S2). Our simulation study shows that attention
394 needs be paid when applying OU models to assess gene expression evolution for
395 phylogenies containing less than 50 taxa.
396



397

398 **Figure 6.** Simulation study for the assessment of parameter estimation bias under an OU-
399 model. The relative bias in estimates of the attraction/selection parameter (α) through
400 1000 simulations under sigma values ranging from 0.1 – 10 and alpha values ranging
401 from 0.01 – 10. Simulations were performed for phylogenies with sizes ranging from 5 to
402 1000 taxa.
403
404

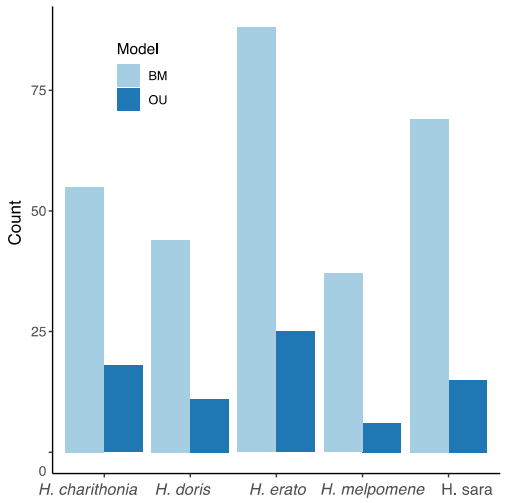
405 *Detection of branch specific shifts in gene expression*

406 To reveal genes whose gene expression patterns have putatively been shaped by
407 directional selection, we tested for branch-specific shifts in evolutionary rates along the
408 *Heliconius* tree. To explore branch-specific shifts in gene expression, firstly we used a
409 BM model to test for the evolutionary rate (σ^2) of a focal branch being different from the
410 background rate (i.e., the rest of the branches in the phylogenetic tree) and assessed
411 significance by applying Bayes factors (Figure S3 and Figure 7). Secondly, we also tested
412 branch-specific shifts through an OU model and tested for a branch-specific shift in gene
413 expression level optimum (θ_F) vs the rest of the tree's θ_B (Figure S4).

414 With a BM approach, we were able to detect a total of 322 branch specific shifts,
415 when considering only tip branches (Figure 7). We found 112 branch specific shifts in the
416 HER linage, 70 in HAS, 67 in HCH, 44 in HDO and 29 in HME (Figure 7 and Figure
417 S4). HCH, HAS and HDO had more shifts towards a down-regulation, although only in
418 HCH and HAS was this difference significant (sign test, HCH: *P*-value 6.738e-05 and
419 HAS: *P*-value 1.653e-06). In HER and HME more up-regulated genes were causing a
420 branch specific shift, although no significant difference was found.

421 When implementing an OU model we recover a total of 75 genes showing a
422 branch specific shift in gene expression optimum (Figure 7 and Figure S4). From these
423 genes, 55 also show a branch specific shift when implementing a BM model and 20 genes
424 show uniquely a gene expression level shift in optimum when using an OU model (Figure
425 S4).

426



427

428 **Figure 7**. Barplot showing branch-specific shifts on gene expression levels in *Heliconius*.
429 Bars in light blue show branch shifts identified by BM and dark blue bars show branch-
430 shifts identified by OU models.

431

432 Next, we assessed within-species gene expression variance of all the genes
433 identified as having a branch-specific shift in gene expression through BM and OU
434 models. When we plotted the distribution of the within-species variance we found that
435 up-regulated genes have a significantly lower variance when compared to genes with a
436 gene expression shift towards a down-regulation (Figure 8). Different evolutionary forces
437 acting on shifts causing up- or down-regulation have the potential of maintaining
438 different levels of gene expression variation within species.

439

440

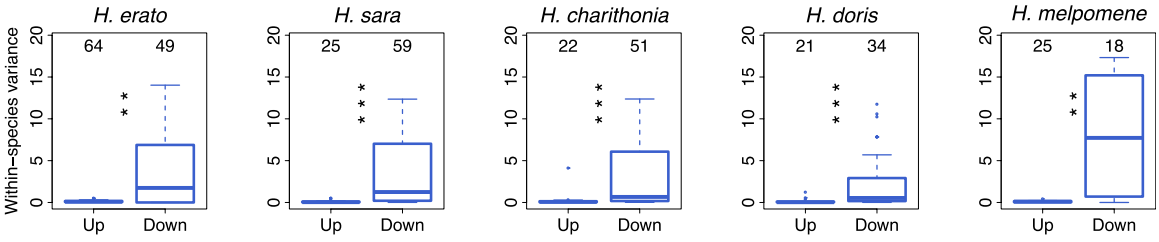


Figure 8. Boxplot showing the distribution of the within-species variance of gene expression levels identified as having a shift towards up-regulation and shifts towards down-regulation. Numbers above the boxplots show the total number of genes identified with a BM and an OU model. Wilcoxon-test: * P<0.05, ** P<0.01, *** P<0.001.

Discussion

Gene expression evolution through genetic drift

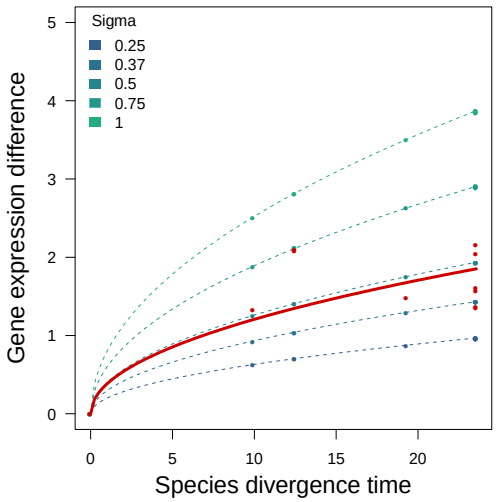
Our study of the evolutionary forces acting on gene expression in eye and brain tissue of *Heliconius* butterflies revealed that most transcriptome levels (81%) are evolving under drift. According to neutral expectations, phenotypic changes are expected to accumulate as a function of time, by drift and mutation alone (Lande 1976). As a consequence, the change of transcriptomic levels through drift should reflect the divergence history of the taxa of interest. From our BM analysis, we conclude that in most of the gene expression levels on eye and brain a phylogenetic signal can be recovered (Figure 3).

Consequently, we hypothesize that gene expression variation influencing phenotypic variation across species mainly arises through random drift. Evolutionary rates of gene expression evolution have been investigated at the population and at the species level and it has been found that the proportion of the type of evolutionary force acting on transcriptomic levels is not constant across taxa (Whitehead and Crawford

462 2006a; Nourmohammad et al. 2017; Stern and Crandall 2018). For example, when
463 examining the evolutionary forces acting on gene expression levels in several fish
464 populations, the authors reported that the dominant force driving expression changes was
465 genetic drift (Whitehead and Crawford 2006b). Comparably, in a study concerning
466 primates, genetic drift was the main force driving gene expression evolution (Khaitovich
467 et al. 2005). The proportion of gene expression levels evolving by drift depends on the
468 strength of natural selection acting on the interrogated transcriptome. For example, in a
469 comparison between different organ types in mammals, gonad gene expression showed
470 the lowest phylogenetic signal when compared to other organs like cerebellum or heart
471 (Brawand et al. 2011). In *Heliconius* butterflies, other organs would need to be tested in
472 order to get a more global understanding on how gene expression is evolving in the
473 whole organism.

474 We explored the gene expression data further by comparing the expected gene
475 expression divergence under a BM model to the observed data. Consequently, we
476 simulated expression levels for 10,000 genes along the known *Heliconius* phylogeny and
477 computed the mean of the pairwise species difference. Similarly, we computed the mean
478 pairwise difference of the observed gene expression data. Alternatively, we can also
479 derive the expected divergence in gene expression levels between two species over time
480 under Brownian motion. Both species evolve under random drift and, thus, their gene
481 expression values are normally distributed with variance σ^2 x T where T is the time since
482 the most recent common ancestor of the two species. Therefore, the difference in gene
483 expression levels between the two species is normally distributed with variance 2 x σ^2 x
484 T. Since we are only interested in the absolute value of the gene expression difference,

485 we use a truncated normal distribution instead. From this truncated normal distribution
486 with mean zero and variance 2 x σ^2 x T we compute the expected gene expression
487 difference through time (Figure 9). For the empirical data, we estimate σ^2 using a sum of
488 squares approach. We find that our observed gene expression data has a close fit to the
489 simulated data (Figure 9).
490



491

492 **Figure 9**. Between species gene expression variance plotted as a function of divergence
493 time according to the *Heliconius* phylogeny. Red: σ^2 from gene expression levels
494 observed in *Heliconius*. Blue: simulated gene expression difference under random drift
495 with different values of sigma.
496

497 *Gene expression evolution through genetic stabilizing selection*

498 Studies done in *Drosophila* and mammals have shown that stabilizing selection is
499 the evolutionary force dominating gene expression evolution (Rifkin et al. 2003; Rohlfs
500 and Nielsen 2015). In contrast to these studies, in *Heliconius* we uncovered that only 3%

of gene expression are either highly conserved (Figure 4) or evolving through stabilizing selection (Figure 5). Factors like tissue type, gene functionality turnover or epistatic levels, have the potential to influence the degree of stabilizing selection acting on the transcriptome (Larracuente et al. 2008; Kalinka et al. 2010; Romero et al. 2012). Additionally, in groups that have experienced an adaptive radiation, like in *Heliconius* (Kozak et al. 2015), and thus have recently experienced an elevated rate of trait evolution, directional selection might be more recurrent than stabilizing selection.

OU models are suitable models to study the force of stabilizing selection acting on a phenotype since the α parameter simulates the strength of selection keeping a trait close to an optimum (Beaulieu et al. 2012), as several studies exemplify (Kalinka et al. 2010; Brawand et al. 2011; Stern and Crandall 2018). When we applied an OU model to identify stabilizing selection on gene expression, we detected parameter estimation biases as shown by our simulation study (Figure 6). For small phylogenies, accurate parameter estimation is challenging since statistical power is weak with small sample sizes (Rohlfs et al. 2014). Specifically, it is very challenging with small phylogenies to distinguish between conserved gene expression levels due to low values of drift (i.e., no change) and high values of selection (i.e., drift is removed due to selection). Not only the number of taxa, but also the depth of the phylogeny can influence the suitability of OU-models to infer stabilizing selection (Fay and Wittkopp 2008; Bedford and Hartl 2009). Therefore, we propose that for small phylogenies, testing for $\sigma^2 = 0$ under a BM framework and assessing for significance by applying Monte Carlo simulations is a better model choice to uncover stabilizing selection. When using this approach, we identified 83 genes with conserved gene expression levels across species. These genes might be involved in

maintaining conserved processes that are essential for the function of eye and brain tissue in *Heliconius*. For example, from the top ten genes with the most conserved gene expression levels, we found the transcription factor *bobby sox* (*bbx*) (Group_674, Appendix 1). BBX, belongs to the high mobility box domain superfamily, which are involved in transcription, replication and chromatin remodeling (Chintapalli et al. 2007). BBX has also been found to have orthologues in flies, human and mice (Nitta et al. 2015) suggesting a high essentiality of *bbx* expression. Another highly conserved orthocluster (Group 977, Appendix 1) was annotated as *glaikit* (Chintapalli et al. 2007), which is known to be essential for the formation of epithelial polarity and nervous system development (Dunlop et al. 2004).

We repeated our previous data exploration by simulating 10,000 genes under an OU-model under a range of σ^2 and α values and computed the mean differences between pairs of species. We observed a reasonably good fit to our data (Figure 10). Most importantly, in Figure 10 we observed a steeper change in gene expression difference between closely related species (low evolutionary distance). Consequently, adding more species, including closely related species, to our model could not only improve OU parameter estimation but could also help in disentangling the evolutionary forces acting on gene expression divergence, specially between closely related species. Interestingly, the observed differences (red dots) in gene expression levels (which are averaged over all 2393 genes) show a clear departure from the expected difference predicted by both a BM and OU model (red lines in Figure 9 and Figure 10). This could be a strong indication of gene expression divergence under different evolutionary forces (drift, stabilizing selection or directional selection).

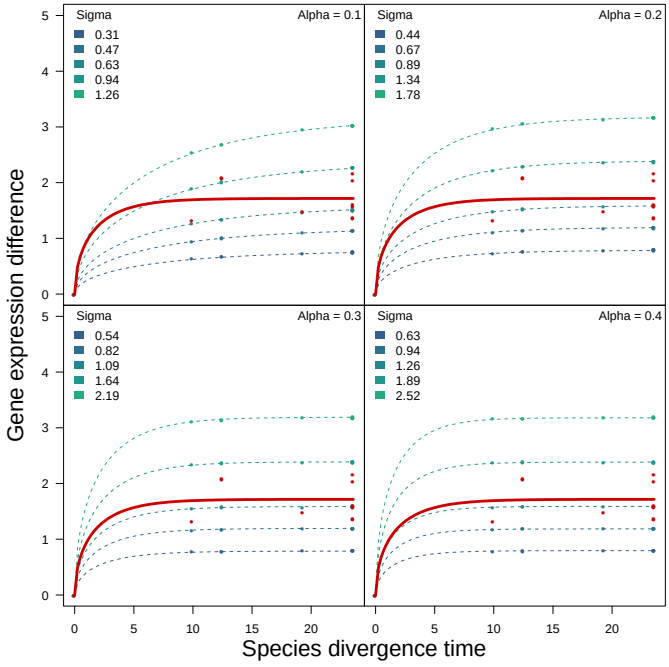


Figure 10. Between species gene expression variance plotted as a function of divergence time according to the *Heliconius* phylogeny. Red: σ^2 from gene expression levels observed in *Heliconius*. Blue: simulated gene expression variance under different values of sigma. Each panel shows estimates for a different value of alpha (α).

Gene expression evolution through genetic directional selection

To reveal branch-specific shifts in gene expression levels we applied BM and OU models, allowing for branch-specific shifts in the rest of the phylogeny. Using this approach, we found that 16% of the genes show a branch-specific shift, towards either up- or down-regulation, with increased expression levels showing lower variance than expected (Figure 8). The direction of gene expression shifts might be influenced by its mode of regulation. For example, in yeast it was found that regulatory mutations

affecting *trans*-regulatory factors were more likely to cause an increase in gene expression. Conversely, mutations on *cis*-regulatory elements were found to be skewed towards a decrease in expression (Metzger et al. 2016). On the other hand, in primates, a higher proportion of species-specific gene expression shifts were found to be towards down-regulation (Khaitovich et al. 2005). If directional selection is causing a branch-specific shift in gene expression one would expect to see a low within-species variance, whereas if the shift is caused by a relaxation of purifying selection or perhaps balancing selection, a higher within-species variance would be expected.

When we looked at the degree of variability between genes showing a shift towards a higher or a lower expression level, we observed that down-regulated genes have a significantly higher variance than genes showing up-regulation (Figure 8). From this observation, we hypothesize that relaxation of purifying selection might be driving the shifts causing down-regulation on gene expression, a pattern which could eventually lead to a loss of expression. However, balancing selection or experimental noise could also lead to an elevated within-species variance. Because of the cost of gene expression, it is expected that only those genes that are essential and have fitness effects will continue to be expressed, whereas genes that are not will eventually stop being transcribed (Stern and Crandall 2018). However, a shift towards down-regulation does not always have to be a consequence of relaxed purifying selection. For example, in the orthocluster with id Group_449_clean_0, a 7-fold lower expression shift was detected in the branch leading to *H. doris* (Figure S5), and a significantly smaller variance than expected transcriptome-wide (Fisher's exact test, *P*-value < 0.001). Directional selection favoring down-regulation of gene expression can occur in a scenario where fine tuning of expression

584 levels are necessary for optimal cell or tissue function (Cayirlioglu et al. 2008; Catalán et
585 al. 2016).

586 On the other hand, genes showing a branch-specific shift towards up-regulation
587 have significantly lower variance when compared to expression level shifts towards
588 down-regulation (Figure 8). This observed pattern could be a result of directional
589 selection acting on gene expression levels leading to a reduction of the variation observed
590 in gene expression. It is possible that in order to achieve an increase in gene expression
591 levels, the selective forces leading to up-regulation would have to be sufficiently strong
592 to result in a greater investment in energy allocated to transcription costs (Wagner 2005;
593 Lang et al. 2009). Some of the genes having the most extreme branch shifts in expression,
594 either toward a higher or a lower expression, are involved in enzymatic activity
595 (Appendix I). Enzymes support biochemical and physiological processes helping in the
596 optimization of tissue function (Wagner and Altenberg 1996; Feller and Gerday 1997).
597 Thus, optimal enzymatic activity might be a key factor for species-specific brain and eye
598 function, which in turn might be optimized for the species-specific life history and
599 ecological environment.

600 A factor possibly influencing the proportion of transcriptome levels found to be
601 evolving through drift, stabilizing or directional selection is the methodology used for
602 orthology assessment. In our analysis of gene expression variation, we assessed variation
603 in orthoclusters where an orthologous hit was found for each of our five *Heliconius*
604 species. Genes with fast-evolving protein rates—to the point that orthology assessment
605 becomes challenging—might also show gene expression shifts, which would not be
606 detectable in our experimental design. For example, orthology assessment for genes

607 showing sex-biased gene expression might require an alternative method. In fact, from
608 the ortholclusters that we identified in this study, only two included genes with sex-
609 biased expression (Catalán et al. 2018). Additionally, gene expression shifts due to
610 duplication events need to be explored by applying an appropriate statistical approach, as
611 expression of genes where a duplication event has happened could contribute to the
612 fraction of the transcriptome evolving by directional selection.

613 With this work, we have generated a set of candidate genes that are putatively
614 evolving through directional selection and that have the potential of being involved in the
615 processes of adaptation and speciation. To test the role of these genes in such processes,
616 functional validation will be necessary to gain a deeper insight in the evolutionary
617 consequences of gene expression shifts. Techniques like *in situ* hybridization, RNAi and
618 CRISPR/Cas9 are adequate tools that can be used in shedding light into the functionality
619 of these genes. Particularly interesting could be those genes whose gene expression levels
620 have shifted to the degree of showing absence of expression (Figure S6). The evolution
621 of gain and loss of gene expression across a phylogeny requires a suitable theoretical
622 framework that should be explored particularly since such events have the potential to
623 accelerate evolution.

624

625 **Acknowledgements**

626 Thanks to Aline Rangel Olguin for technical assistance with the orthocluster analysis.
627 This work was partially supported by National Science Foundation grant IOS-1656260 to
628 A.D.B and by the Knut & Alice Wallenberg Foundation to Jochen Wolf.

629

630 **References**

631 Ahi EP, Richter F, Sefc KM. 2017. A gene expression study of ornamental fin shape in
632 *Neolamprologus brichardi*, an African cichlid species. Sci Rep 7:1–14.
633 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment
634 search tool. J Mol Biol 215:403–410.
635 Ballesteros JA, Hormiga G. 2016. A new orthology assessment method for phylogenomic
636 data: Unrooted Phylogenetic Orthology. Mol Biol Evol 33:1–18.
637 Beaulieu JM, Jhwueng DC, Boettiger C, O'Meara BC. 2012. Modeling stabilizing
638 selection: Expanding the Ornstein-Uhlenbeck model of adaptive evolution.
639 Evolution (N Y) 66:2369–2383.
640 Bedford T, Hartl DL. 2009. Optimization of gene expression by natural selection. Proc
641 Natl Acad Sci U S A 106:1133–1138.
642 Beltrán M, Jiggins C, Brower A, Bermingham E, Mallet J. 2007. Do pollen feeding and
643 pupal-mating have a single origin in *Heliconius*? Inferences from multilocus
644 sequence data. Biol J Linn Soc 92:221–239.
645 Berger JO. 1990. Robust bayesian. J Stat Plan Inference 25:303–328.
646 Brawand D, Soumillon M, Necsulea A, Julien P, Csárdi G, Harrigan P, Weier M, Liechti
647 A, Aximu-Petri A, Kircher M, et al. 2011. The evolution of gene expression levels
648 in mammalian organs. Nature 478:343–348.
649 Butler MA, King AA. 2004. Phylogenetic Comparative Analysis: A Modeling Approach
650 for Adaptive Evolution. Am Nat 164:683–695.
651 Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. 2009. trimAl: A tool for automated
652 alignment trimming in large-scale phylogenetic analyses. Bioinformatics 25:1972–

1973.

Carroll SB. 2005. Evolution at two levels: on genes and form. PLoS Biol 3:e245.

Catalán A, Glaser-Schmitt A, Argyridou E, Duchen P, Parsch J. 2016. An Indel Polymorphism in the *MtnA* 3' Untranslated Region Is Associated with Gene Expression Variation and Local Adaptation in *Drosophila melanogaster*. PLOS Genet 12:1–24.

Catalán A, Macias-Muñoz A, Briscoe AD. 2018. Evolution of Sex-Biased Gene Expression and Dosage Compensation in the Eye and Brain of *Heliconius* Butterflies. Mol Biol Evol 35:2120–2134.

Cayirlioglu P, Kadow IG, Zhan X, Okamura K, Suh GSB, Gunning D, Lai EC, Zipursky SL. 2008. Hybrid neurons in a microRNA mutant are putative evolutionary intermediates in insect CO_2 sensory systems. Science (80-) 510:3–8.

Chen J, Swofford R, Johnson J, Cummings BB, Rogel N, Lindblad-Toh K, Haerty W, Palma F di, Regev A. 2017. A quantitative model for characterizing the evolutionary history of mammalian gene expression. bioRxiv:229096.

Chintapalli VR, Wang J, Dow JAT. 2007. Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. Nat Genet 39:715–720.

Cooper N, Thomas GH, Venditti C, Meade A, Freckleton RP. 2016. A cautionary note on the use of Ornstein Uhlenbeck models in macroevolutionary studies. Biol J Linn Soc 118:64–77.

Dunlop J, Morin X, Corominas M, Serras F, Tear G. 2004. *glaikit* is essential for the formation of epithelial polarity and neuronal development. Curr Biol 14:2039–2045.

Eastman JM, Alfaro ME, Joyce P, Hipp AL, Harmon LJ. 2011. A Novel comparative

676 method for identifying shifts in the rate of character evolution on trees. Evolution (N
677 Y) 65:3578–3589.
678 Enright AJ, Van Dongen S, Ouzounis CA. 2002. An efficient algorithm for large-scale
679 detection of protein families. Nucleic Acids Res 30:1575–1584.
680 Fan Y, Wu R, Chen MH, Kuo L, Lewis PO. 2011. Choosing among partition models in
681 Bayesian phylogenetics. Mol Biol Evol 28:523–532.
682 Fay JC, Wittkopp PJ. 2008. Evaluating the role of natural selection in the evolution of
683 gene regulation. Heredity (Edinb) 100:191–199.
684 Feller G, Gerday C. 1997. Psychrophilic enzymes: Molecular basis of cold adaptation.
685 Cell Mol Life Sci 53:830–841.
686 Felsenstein J. 1985. Phylogenies and the comparative method. Am Nat 125:1–15.
687 Finkbeiner SD, Briscoe AD, Reed RD. 2014. Warning signals are seductive: relative
688 contributions of color and pattern to predator avoidance and mate attraction in
689 Heliconius butterflies. Evolution 68:3410–3420.
690 Finkbeiner SD, Fishman DA, Osorio D, Briscoe AD. 2017. Ultraviolet and yellow
691 reflectance but not fluorescence is important for visual discrimination of
692 conspecifics by *Heliconius erato*. J Exp Biol:jeb.153593.
693 Fitzjohn RG. 2012. Diversitree: Comparative phylogenetic analyses of diversification in
694 R. Methods Ecol Evol 3:1084–1092.
695 Freckleton RP. 2012. Fast likelihood calculations for comparative analyses. Methods
696 Ecol Evol 3:940–947.
697 Gastwirth JL, Gel YR, Miao W. 2009. The Impact of Levene's Test of Equality of
698 Variances on Statistical Theory and Practice. Stat Sci 24:343–360.

699 Glaser-Schmitt A, Parsch J. 2018. Functional characterization of adaptive variation
700 within a *cis*-regulatory element influencing *Drosophila melanogaster* growth. PLoS
701 Biol 16:1–28.
702 Goldman N. 1993. Simple diagnostic statistcal tests of models for DNA substitution. J
703 Mol Evol 36:182–198.
704 Hansen TF. 1997. Stabilizing Selection and the Comparative Analysis of Adaptation.
705 Evolution (N Y) 51:1341.
706 Hastings WK. 1970. Monte Carlo simulation methods using Markov Chains and their
707 applications. Biometrika 57:97–109.
708 Ho LST, Ané C. 2014. Intrinsic inference difficulties for trait evolution with Ornstein-
709 Uhlenbeck models. Methods Ecol Evol 5:1133–1146.
710 Höhna S, Landis MJ, Heath TA. 2017. Phylogenetic inference using RevBayes. Curr
711 Protoc Bioinforma 2017:6.16.1-6.16.34.
712 Höhna S, Landis MJ, Heath TA, Boussau B, Lartillot N, Moore BR, Huelsenbeck JP,
713 Ronquist F. 2016. RevBayes: Bayesian phylogenetic inference using graphical
714 models and an interactive model-specification language. Syst Biol 65:726–736.
715 Ives AR, Midford PE, Garland T. 2007. Within-species variation and measurement error
716 in phylogenetic comparative methods. Syst Biol 56:252–270.
717 Kalinka AT, Varga KM, Gerrard DT, Preibisch S, Corcoran DL, Jarrells J, Ohler U,
718 Bergman CM, Tomancak P. 2010. Gene expression divergence recapitulates the
719 developmental hourglass model. Nature 468:811–814.
720 Kass KE, Raftery AE. 1995. Bayes Factors. J Am Stat Assoc 90:773–795.
721 Katoh K, Toh H. 2008. Recent developments in the MAFFT multiple sequence alignment

722 program. Brief Bioinform 9:286–298.

723 Khaitovich P. 2005. Parallel patterns of evolution in the genomes and transcriptomes of

724 humans and chimpanzees. Science (80-) 309:1850–1854.

725 Khaitovich P, Pääbo S, Weiss G. 2005. Toward a neutral evolutionary model of gene

726 expression. Genetics 170:929–939.

727 Khaitovich P, Weiss G, Lachmann M, Hellmann I, Enard W, Muetzel B, Wirkner U,

728 Ansorge W, Pääbo S. 2004. A neutral model of transcriptome evolution. PLoS Biol

729 2.

730 Kozak KM, Wahlberg N, Neild AFE, Dasmahapatra KK, Mallet J, Jiggins CD. 2015.

731 Multilocus species trees show the recent adaptive radiation of the mimetic

732 *Heliconius*. butterflies. Syst Biol 64:505–524.

733 Kronforst MR, Papa R. 2015. The functional basis of wing patterning in *Heliconius*

734 butterflies: The molecules behind mimicry. Genetics 200:1–19.

735 Lande R. 1976. Natural Selection and Random Genetic Drift in Phenotypic Evolution.

736 Evolution (N Y) 30:314–334.

737 Lang GI, Murray AW, Botstein D. 2009. The cost of gene expression underlies a fitness

738 trade-off in yeast. Proc Natl Acad Sci 106:5755–5760.

739 Larracuente AM, Sackton TB, Greenberg AJ, Wong A, Singh ND, Sturgill D, Zhang Y,

740 Oliver B, Clark AG. 2008. Evolution of protein-coding genes in Drosophila. Trends

741 Genet 24:114–123.

742 Lee G, Foss M, Goodwin SF, Carlo T, Taylor BJ, Hall JC. 2000. Spatial, temporal, and

743 sexually dimorphic expression patterns of the fruitless gene in the *Drosophila*

744 central nervous system. J Neurobiol 43:404–426.

McCulloch KJ, Yuan F, Zhen Y, Aardema ML, Smith G, Llorente-Bousquets J, Andolfatto P, Briscoe AD. 2017. Sexual Dimorphism and Retinal Mosaic Diversification Following the Evolution of a Violet Receptor in Butterflies. Mol Biol Evol 34:1–14.

Mendoza-Cuenca L, Macías-Ordóñez R. 2005. Foraging polymorphism in Heliconius charitonia (Lepidoptera: Nymphalidae): morphological constraints and behavioural compensation. J Trop Ecol 21:407–415.

Merrill RM, Dasmahapatra KK, Davey JW, Dell'Aglio DD, Hanly JJ, Huber B, Jiggins CD, Joron M, Kozak KM, Llaurens V, et al. 2015. The diversification of *Heliconius* butterflies: What have we learned in 150 years? J Evol Biol 28:1417–1438.

Metropolis N, Rosenbluth AW, Rosenbluth MN, Teller AH, Teller E. 1953. Equation of state calculations by fast computing machines. J Chem Phys 21:1087–1092.

Metzger BPH, Duveau F, Yuan DC, Tryban S, Yang B, Wittkopp PJ. 2016. Contrasting Frequencies and Effects of *cis*- and *trans*-Regulatory Mutations Affecting Gene Expression. Mol Biol Evol 33:1131–1146.

Montgomery SH, Merrill RM, Ott SR. 2016. Brain composition in *Heliconius* butterflies, posteclosion growth and experience-dependent neuropil plasticity. J Comp Neurol 524:1747–1769.

Nadeau NJ. 2016. Genes controlling mimetic colour pattern variation in butterflies. Curr Opin Insect Sci 17:24–31.

Nitta KR, Jolma A, Yin Y, Morgunova E, Kivioja T, Akhtar J, Hens K, Toivonen J, Deplancke B, Furlong EEM, et al. 2015. Conservation of transcription factor binding specificities across 600 million years of bilateria evolution. Elife 2015:1–20.

768 Nourmohammad A, Rambeau J, Held T, Kovacova V, Berg J, Lässig M. 2017. Adaptive
769 evolution of gene expression in *Drosophila*. Cell Rep 20:1385–1395.
770 O'Meara BC, Ané C, Sanderson MJ, Wainwright PC. 2006. Testing for different rates of
771 continuous trait evolution using likelihood. Evolution (N Y) 60:922–933.
772 Punta M, Coggill P, Eberhardt R, Mistry J, Tate J, Boursnell C, Pang N, Forslund K,
773 Ceric G, Clements J, et al. 2012. The Pfam protein families databases. Nucleic Acids
774 Res 30:1–12.
775 Rifkin SA, Kim J, White KP. 2003. Evolution of gene expression in the *Drosophila*
776 *melanogaster* subgroup. Nat Genet 33:138–144.
777 Rohlfs R V., Harrigan P, Nielsen R. 2014. Modeling gene expression evolution with an
778 extended Ornstein-Uhlenbeck process accounting for within-species variation. Mol
779 Biol Evol 31:201–211.
780 Rohlfs R V., Nielsen R. 2015. Phylogenetic ANOVA: The expression variance and
781 evolution model for quantitative trait evolution. Syst Biol 64:695–708.
782 Romero IG, Ruvinsky I, Gilad Y. 2012. Comparative studies of gene expression and the
783 evolution of gene regulation. Nat Rev Genet 13:505–516.
784 Salcedo C. 2010. Environmental Elements Involved in Communal Roosting in *Heliconius*
785 Butterflies (Lepidoptera: Nymphalidae). Environ Entomol 39:907–911.
786 Signor SA, Nuzhdin S V. 2018. The evolution of gene expression in *cis* and *trans*. Trends
787 Genet:1–13.
788 Sinharay S, Stern HS. 2002. On the sensitivity of Bayes factors to the prior distributions.
789 Am Stat 56:196–201.
790 Stamatakis A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic

791 analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690.
792 Stern DB, Crandall KA. 2018. The Evolution of Gene Expression Underlying Vision
793 Loss in Cave Animals. Mol Biol Evol 35:2005–2014.
794 Uyeda JC, Harmon LJ. 2014. A novel Bayesian method for inferring and interpreting the
795 dynamics of adaptive landscapes from phylogenetic comparative data. Syst Biol
796 63:902–918.
797 Wagner A. 2005. Energy constraints on the evolution of gene expression. Mol Biol Evol
798 22:1365–1374.
799 Wagner GP, Altenberg L. 1996. Complex Adaptations and the Evoluation of
800 Evolvability. Evolution (N Y) 50:967–976.
801 Walters JR, Stafford C, Hardcastle TJ, Jiggins CD. 2012. Evaluating female remating
802 rates in light of spermatophore degradation in *Heliconius* butterflies: Pupal-mating
803 monandry versus adult-mating polyandry. Ecol Entomol 37:257–268.
804 Wanner KW, Anderson AR, Trowell SC, Theilmann DA, Robertson HM, Newcomb RD.
805 2007. Female-biased expression of odourant receptor genes in the adult antennae of
806 the silkworm, *Bombyx mori*. Insect Mol Biol 16:107–119.
807 Warnefors M, Eyre-Walker A. 2012. A selection index for gene expression evolution and
808 its application to the divergence between humans and chimpanzees. PLoS One 7.
809 Whitehead A, Crawford DL. 2006a. Variation within and among species in gene
810 expression: Raw material for evolution. Mol Ecol 15:1197–1211.
811 Whitehead A, Crawford DL. 2006b. Neutral and adaptive variation in gene expression.
812 Proc Natl Acad Sci 103:5425–5430.
813 Wong ES, Thybert D, Schmitt BM, Stefflova K, Odom DT, Flicek P. 2015. Decoupling

814 of evolutionary changes in transcription factor binding and gene expression in

815 mammals. Genome Res 25:167–178.

816