Article: Discoveries

Mapping selection within *Drosophila melanogaster* embryo's anatomy

Irepan Salvador-Martínez[†,1,2], Marta Coronado-Zamora[†,3], David Castellano[†,4], Antonio Barbadilla[*,3] and Isaac Salazar-Ciudad[*,1,3]

Author affiliations:

[1] Evo-devo Helsinki community, Centre of Excellence in Experimental and Computational Developmental Biology, Institute of Biotechnology, University of Helsinki, Helsinki, Finland

[2] Current address: Department of Genetics, Evolution and Environment. University College London, WC1E 6BT, UK

[3] Genomics, Bioinformatics and Evolution. Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain

[4] Bioinformatics Research Centre, Aarhus University, Aarhus, Denmark

[†] **Co-first author**

[*] **Corresponding authors**:

Antonio Barbadilla, e-mail: antonio.barbadilla@uab.cat (AB)

Isaac Salazar-Ciudad, e-mail: isalazar@mappi.helsinki.fi (ISC)

Abstract

We present a survey of selection across *Drosophila melanogaster* embryonic anatomy. Our approach integrates genomic variation, spatial gene expression patterns and development to map adaptation over the entire embryo's anatomy. Our adaptation map is based in analyzing gene expression spatial information for 5,969 genes (from text-based annotations of in situ hybridization data directly from the BDGP database, Tomancak et al. 2007) and polymorphism and divergence in these genes (from the project DGRP, Mackay et al. 2012).

The proportion of non-synonymous substitutions that are adaptive, neutral or slightly deleterious are estimated for the set of genes expressed in each embryonic anatomical structure using the DFE-alpha method (Eyre-Walker and Keightley 2009), a robust derivative of the McDonald and Kreitman test (McDonald and Kreitman 1991). We also explore whether different anatomical structures differ in the phylogenetic age, codon usage or expression bias of the genes they express and whether genes expressed in many anatomical structures show more adaptive substitutions than other genes.

We found that: (i) most of the digestive system and ectoderm-derived structures are under selective constraint, (ii) the germ line and some specific mesoderm-derived structures show high rates of adaptive substitution and (iii), the genes that are expressed in a small number of anatomical structures show higher expression bias, lower phylogenetic ages and less constraint.

Keywords

Adaptation, selective constraint, natural selection, evo-devo, DFE-alpha, codon usage, phylogenetic age, expression bias, embryo anatomy, Drosophila, gene expression

Introduction

Adaptation is a core concept of biological evolution and its measurement is an important pursuit of research in evolutionary biology. Adaptation can be studied both at the phenotypic and genotypic level. One can, for example, measure the distribution of a phenotypic trait in a population, study the performance of each trait value in the lab, or in the wild, and infer which proportion of the observed changes over generations is attributable to adaptive natural selection (Hereford et al. 2004). In practice, measuring adaptation directly on phenotypic traits in the wild is challenging and time consuming and therefore most studies on phenotypic adaptation are limited to a single or a small number of traits per organism (Hereford et al. 2004). Easily identifiable and measurable phenotypic traits that are known, or suspected, to be adaptive are preferentially chosen in these studies (Hereford et al. 2004), which may bias our view on how the whole phenotype of an organism evolves. To our knowledge, no measurement of natural selection over the whole body morphology has ever been attempted.

The relationship between genetic and phenotypic variation is well known to be complex (Alberch 1982; Salazar-Ciudad 2007). In spite of that it can be expected that, at least at a statistical level, adaptation in a body part can be inferred from adaptation in the genes it expresses. In here, we take such approach to build a map of adaptation over the embryo's anatomy.

In current population genomics research, adaptive (positive) selection and other selection regimes can be inferred and measured studying the pattern of genome variation within and between species. A growing number of sophisticated statistical methods has been developed to detect and measure selection at the DNA level for an increasing number of genome-wide variation data and species (reviewed by Vitti et al. 2013; Casillas and Barbadilla 2017). Most of these methods assume that selection at the DNA level leaves a distinctive footprint on the patterns of genetic variation when compared with the variation patterns expected under a null (neutral) hypothesis of absence of selection (Kimura 1968). From 30 to 50% of fixed non-synonymous mutations in *D. melanogaster* are estimated to be adaptive (Casillas and Barbadilla 2017). Is this amount of molecular adaptation randomly distributed with respect to different phenotypic traits such as organs or morphological body parts? We are going to address this question by measuring the action of natural selection in genes specifically expressed across development .

A widely-used statistic to infer selection on coding DNA sequences is the ratio of non-synonymous to synonymous substitution per site (K_a/K_s, d_N/d_S or ω), which uses sequence divergence data between species. If in a given sequence $K_a/K_s < 1$ then the

sequence is under constraint since it has less non-synonymous substitutions than expected from a neutral setting. Given that most non-synonymous mutations are deleterious, the K_a/K_s statistics has little power to detect positive selection, that is, an excess of non-synonymous substitutions respect to the expected ones. The Ka/Ks ratio, thus, conflates the contributions of adaptive and neutral changes to sequence divergence; a high K_a/K_s ratio could reflect little constraint, or a combination of adaptation and purifying selection (Sella et al. 2009). Accordingly, the ratio K_a/K_s should mainly be considered as a measurement of selective constraint. A more powerful approach is combining genomic polymorphism data (variation within a species) and divergence (variation between species), as does the McDonald-Kreitman test (MKT) (McDonald and Kreitman 1991). Unlike the K_a/K_s ratio, the MKT normalizes the divergence ratio (K_a/K_s) with the polymorphism ratio (π_a/π_s), which allows taking into account the constraint on non-synonymous sites and, thus, increase the power of detection of positive selection ((K_a/K_s)/(π_a/π_s) > 1). MKT allows also quantifying the proportion of fixed variants that are adaptive (α) and the rate of these adaptive substitutions relative to the mutation rate, ω_α (as $\alpha \times K_a/K_s$, Gossmann et al. 2010).

In this study, we use the DFE-alpha method (Eyre-Walker and Keightley 2009) to estimate α and ω_α. The DFE-alpha method is a robust derivative of MKT. Since adaptive mutations tend to be fixed quickly (Kimura 1957), they will rarely be detected as polymorphic variants but only as a divergent site (that is once fixed, Hudson et al. 1987; McDonald and Kreitman 1991; Sawyer and Hartl 1992; Hurst 2002). Accordingly, adaptation in the genome of a species is inferred when there is an excess of non-synonymous divergence relative to its non-synonymous polymorphism. The divergence and polymorphism in synonymous sites, which are assumed to be neutral, are used to estimate the underlying mutation rate and the expected polymorphism and divergence under a neutral scenario. However, the estimation of α can be biased due to the segregation of slightly deleterious non-synonymous mutations (Eyre-Walker 2002). Given a stable population size, α is underestimated under slightly deleterious mutations because they tend to contribute more to polymorphism than to divergence. The DFE-alpha method corrects for this possible bias by first estimating the Distribution of Fitness Effects (DFE) of mutations from DNA sequence polymorphism data at selected sites by a gamma distribution and then calculating how many non-adaptive substitutions are expected to become fixed given the DFE inferred from polymorphism data. Thus, as explained above, any excess of non-synonymous substitutions should be attributable to adaptation.

Several studies using genomics have estimated adaptation in embryonic development. There is, for example, a large body of literature estimating the K_a/K_s ratio and related

measures to uncover different selection regimes in genes with different biochemical or physiological functions (as defined by the GO terms) (Rocha and Danchin 2004; Salathe et al. 2006; Hanada et al. 2007; Zhang et al. 2007; Yang et al. 2012; Zhao et al. 2013; Yang et al. 2014). or in genes expressed in different developmental stages (Davis et al. 2005; Roux and Robinson-Rechavi 2008; Artieri et al. 2009; Kalinka et al. 2010; Mensch et al. 2013; Piasecka et al. 2013). There are also several studies on genes' phylogenetic age and evolution and development. The phylogenetic age of a gene is the phylogenetic level at which homologues for a gene are found (e.g., if a gene has homologues among eukaryota the phylogenetic age is larger than if the gene has homologues only among Drosophilids). Thus, it has been found that genes with different evolutionary ages differ in their genomic properties (older genes tend to be longer, highly expressed, with larger intron density and more constrained; (Wolf et al. 2009). Other studies have also found a relationship between the rate of non-synonymous substitutions in a gene and its expression bias, the level of restriction of its expression in developmental time (Duret and Mouchiroud 2000; Subramanian and Kumar 2004; Wright et al. 2004), codon usage (Sharp 1991; Marais et al. 2004; Rocha and Danchin 2004) and expression level (Pal et al. 2001; Subramanian and Kumar 2004; Drummond et al. 2005; Lemos et al. 2005).

This study differs in two main aspect from previous ones jointly analyzing evolutionary genomics and development: (1) it is focused in space (embryo's anatomy) instead of time (developmental stages) and (2) since it is a population genomics analysis, it is able to measure the rate of adaptive substitution (ω_α) and not just the rate of non-synonymous substitutions (as in the K_a/K_s ratio). Thus, our aim is to estimate and compare both adaptation and selective constraint through the body of *D. melanogaster*. The time scale spans around 7.4 MY (Tamura et al. 2004), from the present to the most recent common ancestor of *D. melanogaster* and *D. yakuba*, the outgroup species used in this study to estimate divergence. Adaption and constraint is estimated for six different embryonic stages, from maternal stage 1-3 to stage 13-16, a stage shortly before the larva hatches. As gene expression in the last embryonic stage analyzed (the embryo-larva transition) closely resembles that of the larva stages (Arbeitman et al. 2002) and no much cell movement or massive changes in gene expression are known to occur at this stage (Hartenstein 1993), our analysis should inform also about adaptation over the larval body. We also study whether there is a relationship between the number of anatomical structures in which a gene is expressed (that can be seen as a measure of pleiotropy in the space of the embryo) and ω_α. In addition, we measure the phylogenetic age, expression bias and codon usage for the different anatomical

structures and explore whether there is an association between these genomic variables and selection during development.

Our morphological adaptation map is based in the analyses of spatial patterns of gene expression for 5,969 genes (from text-based annotations of in situ hybridization data directly from the BDGP database, Tomancak et al. 2007) and polymorphism and divergence in these genes (from the DGRP project, Mackay et al. 2012). We first collected the lists of genes expressed in each different embryonic anatomical structure considered in the BDGP (15 anatomical structures for which the expression data was described in Tomancak et al. 2007; see Supplementary Table 5 for these). The polymorphism and divergence (out of *D. yakuba*) of the genes in each list were analyzed together (genes in each list were concatenated for the analysis) with the DFE-alpha method (Eyre-Walker and Keightley 2009). With this method, we estimate for measures of selection: (i) α (ii) the standard measure of selective constraint, ω (K_a/K_s), (iii) ω_α and (iv) ω_d, the rate of non-adaptive (neutral or slightly deleterious) substitutions relative to the rate of neutral substitutions. The rate of synonymous substitutions was estimated following two different approaches. In the first one we use the 4-fold degenerated sites. In the second approach, we use instead the short intron sites in each gene (Halligan and Keightley 2006). The analysis based on short introns has the advantage of filtering out the possible effect of codon usage (reviewed by Hershberg and Petrov 2008) but in our dataset, only half of the analyzed genes have short introns adequate for the analysis. The results of both analyses are largely consistent (see below). We have also calculated whether the observed ω, ω_α and ω_d values are higher (or lower) than those expected by chance alone. For that we performed a permutation test in which we assigned genes (from our dataset) to each anatomical structure at random and recalculated ω, ω_α and ω_d (while keeping the number of genes per anatomical structure and the number of genes co-expressed between anatomical structures as in the original data). We then compared the observed ω, ω_α and ω_d values with those found by repeating this permutation test many times. We also performed another statistical test, a Student's t statistical, in which we simply compare the ω, ω_α and ω_d for the set of genes expressed in an anatomical structure and the set of genes that are not express in such anatomical structure. These two statistical tests give very similar results (see Supplementary Tables 7 and 14). See Material and methods for details on the data analyzed, its processing and statistical analysis.

Results

Selection at the germ layer level

First, we searched for differences in the selective regimes experienced by the tissues derived from each of three primary germ layers of the *Drosophila* embryo. These germ layers constitute the first three tissues in embryonic development: ectoderm, mesoderm and endoderm. Later embryonic and larval tissues develop from one of such germ layers. We analyzed the set of genes that are exclusively expressed in the derivatives of each germ layer, that is, those genes whose expression overlapped for two or three layers were excluded from the analysis. The number of genes analyzed for each germ layer is provided in Supplementary Table 1.

We found that the set of genes exclusively expressed in the ectoderm-derived tissues are more constrained than those expressed in the other two layers (low ω, 2-tailed permutation test, $p = 0.004$). On the other hand, the set of genes expressed in the tissues derived from the mesoderm show higher rates of adaptive substitutions (high ω_α, 2-tailed permutation test, $p < 0.001$). Finally, the set of genes expressed exclusively in the tissues derived from the endoderm, show a relative relaxation of selection compared to the other two layers (high ω_d, 2-tailed permutation test, $p = 0.046$).

Neither recombination rates, nor gene density nor mutation rates differ between the genes expressed in each germ layer. Hence, these genome variables do not seem to bias our measurements of differential selection (ANOVA analysis, Supplementary Tables 2-4). See Material and methods for details.

Selection at the anatomical structure level

We analyzed the set of genes expressed in each anatomical structure reported in the BDGP (Tomancak et al. 2007). A gene was counted as expressed in a given anatomical structure if it was expressed in in at least one developmental stage of the structure. The studied anatomical structures were (Supplementary Figure 1): “Amnioserosa/Yolk”, “Procephalic Ectoderm/CNS”, “Peripheral Nervous System (PNS)”, “Foregut”, “Ectoderm/Epidermis”, “Tracheal System”, “Salivary Gland”, “Hindgut/Malpighian tubules”, “Mesoderm/Muscle”, “Head Mesoderm/Circulatory”, System/Fat body”, “Garland cells/Plasmatocytes/Ring gland”, “Germ line” and “Endoderm/Midgut”. In addition, we also analyzed the genes that are expressed

ubiquitously or that are present already in the egg. These latter genes were categorized following the original BDGP database (Tomancak et al. 2007), either as “Ubiquitous” or “Maternal”. The number of genes per anatomical structure can be found in Supplementary Table 5.

We found that all four selective regimes analyzed vary through the embryo anatomy (see Supplementary Figure 2 for a schematic illustration of the results). The genes expressed in the anatomical structure “Garland cells/Plasmatocytes/Ring gland” and these expressed in the “Germ line” exhibited high rates of adaptive substitution (higher than the expected rate in random permutations of the genes in the database: high ω_α, 2-tailed permutation test, p = 0.018, high ω_α, 2-tailed permutation test, p = 0.018, respectively). The same was found for those genes expressed in the “Head mesoderm/Circulatory system/Fat body”, but only with a marginal significance (high ω_α, 2-tailed permutation test, p = 0.052).

Contrastingly, several anatomical structures of the digestive system exhibit a high constraint in the genes they express (higher than expected from the permutation test). This is the case of the “Foregut” (low ω, 2-tailed permutation test, $p < 0.001$, low ω_d, p = 0.012), the “Hindgut/Malpighian tubules” (low ω, 2-tailed permutation test, $p < 0.001$, low ω_d, p = 0.018), the “Endoderm/Midgut” (low ω, 2-tailed permutation test, $p < 0.001$, low ω_d, p = 0.018, low ω_α, p = 0.024) and the “Salivary gland” (low ω, 2-tailed permutation test, $p < 0.001$). In several neuroectodermic anatomical structures the set of genes expressed showed also higher selective constraint than expected by chance alone. This is the case of the “Peripheral Nervous System” (PNS) (low ω, 2-tailed permutation test, p < 0.001, low ω_α, p = 0.016) and the “Procephalic Ectoderm/CNS” (low ω, 2-tailed permutation test, p = 0.004, low ω_d, $p < 0.001$). Higher constraint was also found in the “Ectoderm/Epidermis” (low ω, 2-tailed permutation test, $p < 0.001$, low ω_d, p = 0.030, low ω_α, p = 0.024), and the “Mesoderm/Muscle” (low ω, 2-tailed permutation test, p = 0.016).

Finally, “Maternal” genes exhibit higher values of relaxed selection (high ω_d, 2-tailed permutation test, p = 0.026) while the set of genes in the anatomical structures “Ubiquitous” and “Amnioserosa” genes do not show evidence of any preferential regime of selection. Very similar results were found when short introns, instead of 4-fold degenerated sites, were used to estimate the mutation rate (Supplementary Table 6) and when a Student’s t statistical test was used instead of the permutation test (Supplementary Table 7). Neither recombination rate, nor gene density nor mutation rate differ between the genes expressed in each anatomical structure (ANOVA analysis, Supplementary Tables 8-10).

Analysis by stages

The previous anatomical structures were further analyzed by splitting them between stages. In other words, each set of genes expressed in an anatomical structure and stage were analyzed independently (even for the genes expressed in the same anatomical structure at some other stage). We analyzed a total of six developmental stages than span the first 16 hours of the embryo development: stage 1 (1-3h), stage 2 (4-6h), stage 3 (7-8h), stage 4 (9-10h), stage 5 (11-12h) and stage 6 (13-16h). The list of genes analyzed by anatomical structure and developmental stage can be found in Supplementary Table 11. Figure 1 shows the results obtained in this analysis. Supplementary Table 12 shows the p-values of the permutation tests for each anatomical structure, and Supplementary Figure 3 a schematic illustration of the results. In general, the results are very similar to the ones in the previous section. The anatomical structures in stage 13-16 (an embryonic stage close to the larva stage) are the ones that most often exhibit ω_α and ω values that are significantly different from the ones expected by chance alone. We find evidence of relaxation of selection on the first stage, where maternal genes are expressed. Very similar results were found for most of the anatomical structures when short introns were used to estimate the mutation rate (Supplementary Table 13) and when using the Student's t statistical test instead of the permutation test (Supplementary Table 14).

Relationship between phylogenetic age, *Fop*, expression bias, and adaptation

We also analyzed the relationship between the phylogenetic age (using Drost 2014 data), the expression bias and expression level (using modENCODE RNA-seq expression data, Graveley et al. 2011), the frequency of optimum codons (*Fop*) and the different selection regimes. The expression bias index indicates how temporally restricted is the expression of a gene during embryonic development. For a given gene, an expression bias value of 1 means that it is only expressed in one developmental stage, whereas a value of 0 means that it is expressed in all developmental stages (see Material and methods). The frequency of optimum codons also ranges from 0 to 1, 0 indicates that no optimum codon is present in the sequence while 1 means that all the codons used are the optimal ones.

We took the set of genes expressed in the anatomical structures and divided them in 5 equally sized categories (except for phylogenetic age, where categories were manually defined) depending on their phylogenetic age (Supplementary Figure 4A). We did a similar categorization of genes by expression bias levels (Supplementary Figure 4D),

expression level (Supplementary Figure 4G) and *Fop* (Supplementary Figure 4J). See Supplementary Tables 15-18 for the quantile categories.

The measurements of selection on the set of genes with distinct ages, *Fop,* expression level and expression bias differ significantly. Supplementary Figure 4B and 3C show that older genes (categories 1 and 2) have higher constraint and lower rates of adaptive substitution (Supplementary Figure 4B and 4C). A very similar trend is found for expression bias (Supplementary Figure 4E and 4F), with less biased genes showing more constraint and lower rates of adaptive substitution. The contrary is found for expression level (Supplementary Figure 4H and 4I) (measured as the logarithm of the maximum expression in RPKM levels using modENCODE RNA-seq in *D. melanogaster*) and *Fop* (Supplementary Figure 4K and 4L). Selective constraint decreases with both *Fop* and the expression level and the rate of adaptive substitutions decreases with *Fop*. As shown in Supplementary Figure 5, the anatomical structures with the highest rates of adaptive substitution are not the anatomical structures with the lowest *Fop*, newest genes or highest expression bias. Therefore, these variables do not seem to explain the differences in the rates of adaptive substitution found between anatomical structures.

Relationship between phylogenetic age, *Fop* and expression bias

To acquire a better understanding of the results in the previous section, we also analyzed the relationship between the phylogenetic age, expression bias and frequency of optimum codons (*Fop*) of the genes analyzed.

We found a positive correlation between phylogenetic age and expression bias (Figure 2) (Pearson's ρ = 0.490, p = 0.039). Thus, genes that are younger phylogenetically tend to be expressed in more specific stages than phylogenetically older genes, which are more broadly expressed through stages. Furthermore, genes expressed in anatomical structures derived from the endoderm are phylogenetically the oldest on average whilst those derived from the ectoderm express the most phylogenetically recent genes (except for the set of genes expressed in the salivary glands). The "Segmental/GAP" anatomical structure is also exceptional in expressing the newest genes (note that during development these genes are expressed before the germ layers are formed). A negative correlation is found between phylogenetic age and *Fop* (Pearson's ρ = -0.698, $p = 1.27 \times 10^{-3}$). The salivary glands also stand out for having one of the highest *Fop* values.

Relationship between spatial pleiotropy, phylogenetic age, expression bias and adaptation

Finally, the set of genes expressed in the 18 anatomical structures were divided in eight groups depending on the number of anatomical structures in which they are expressed (1, 2, …, 7, 8 or more, see Supplementary Figure 6). These values can be taken as a rough measurement of the pleiotropic effects of a gene on embryonic anatomy and we call this index spatial pleiotropy.

We first analyzed the relationships between the eight groups and the phylogenetic age, *Fop* and expression bias. For that we resampled 100 times with replacement the genes of each group. Results are shown in the Figure 3. We found a negative correlation between the number of anatomical structures in which a gene is expressed and its phylogenetic age (Figure 3A, Pearson's ρ: 0.777, p = 0.0233) and expression bias (Figure 3B, Pearson's ρ: -0.900 p = 0.002) and a positive correlation with *Fop* (Figure 3C, Pearson's ρ: 0.926, $p = 9.51\times10^{-4}$)

When we analyzed the evidences of selection in each group of genes, we found a negative correlation between spatial pleiotropy and both ω (Figure 4A, Pearson's ρ: -0.890, p = 0.003) and ω_d (Figure 4B, Pearson's ρ: -0.749, p = 0.032). Thus, genes expressed in a low number of anatomical structures seem to be, on average, less selectively constrained than genes expressed in a high number of anatomical structures. No correlation was found between ω_a and the number of anatomical structures in which a gene is expressed (Figure 4C) and, as shown in Supplementary Figure 5D, the anatomical structures with the highest ω_a are not the ones where the genes are expressed in the smallest number of other anatomical structures (so again our results are not simply explainable from differences in these variables among anatomical structures).

Discussion

This work measures which parts of the embryo's body exhibit significantly higher ω_α, ω_d and ω values and which exhibit significantly lower ω_α, ω_d and ω values in the genes they express compared to the rest of genes expressed in the other anatomical structures of the embryo. The anatomical structures with high ω_α, values should be interpreted as body regions with high rates of adaptive substitution for the amino acid sequences of the gene products they express, while the anatomical structures with low ω_α values should be interpreted as body regions where such adaptive changes have barely occurred from the current populations of *D. melanogaster* to its most recent common ancestor with *D. yakuba*. The anatomical structures with high ω and ω_d, values, instead, should be interpreted as body regions under relaxed natural selection, while the anatomical structures with low ω and ω_d values should be interpreted as body regions under a history of selective constraint.

The latest embryonic stage analyzed, stage 13-16, shows the most contrasting values of ω_α and ω between anatomical structures. In this stage, the anatomical structures and the gene co-expression spatial patterns, are positioned and shaped in very similar ways than in those of the larva (no major morphogenetic rearrangements occur from that stage onwards, (Hartenstein 1993)). In that sense, the results in this latest stage could be taken as a proxy for adaptation over the body parts of the functional larva.

In summary, we found high rates of adaptive substitution in the "Germ line", the "Garland cells/Plasmatocytes/Ring gland" and perhaps also the "Head mesoderm/Circulatory system/Fat body". Most of the rest of the body seems under selective constraint. Our results are consistent with previous results from other non-development studies. Thus, our results of adaptation in the "Germ line" are consistent with previously reported high K_a (Civetta and Singh 1995; Wyckoff et al. 2000; Meiklejohn et al. 2003; Pröschel et al. 2006; Haerty et al. 2007; Assis et al. 2012) and α (Pröschel et al. 2006) in testis- or sperm-specific genes and sperm-related genes

already expressed in germ line cells (Civetta et al. 2005; Bauer DuMont et al. 2007). The category “Garland cells/Plasmatocytes/Ring gland” is closely linked to the immune system. Plasmatocytes conform the 95% of all the immune cells in *D. melanogaster* (similar to our macrophages, (Ratheesh et al. 2015) and the ring gland has been also related to the immune system (Christesen et al. 2016). The immune system has already been shown to exhibit high rates of adaptive substitution, high α, in *D. melanogaster (Schlenke and Begun 2003; Jiggins and Kim 2007; Obbard et al. 2009; Early et al. 2017)*.

Overall, our results suggest that there is a high degree of conservation over the genes expressed over most embryonic anatomy and some degree of adaptive substitutions in the set of genes expressed in anatomical structures involved in reproduction and immunity.. We can state that selective constraint is pervasive over most of the embryo’s anatomy except for anatomical structures that show evidence of adaptation in the embryo (sperm-related genes expressed in germ line cells) but in the adult too (immune system and sperm-related genes).

Our analysis does not explain why some specific anatomical structures show high rates of adaptive substitutions but helps in discarding some simple explanations. Thus, for example, the high ω_α in some anatomical structures does not seem to be related to the expression of genes with low levels of pleiotropy in those anatomical structures, at least as compared to the genes expressed in other anatomical structures (Supplementary Figure 5D). Genes with a high expression bias influence developmental processes in a restricted time window while genes expressed in few anatomical structures affect only the developmental processes in those anatomical structures. In both cases, a gene is affecting only a small number of processes and, thus, it can be said to have a low level of pleiotropy. Very pleiotropic genes have reiteratively been suggested to change more slowly in evolution than genes with little pleiotropy. This is because the more processes a gene influences the more unlikely it is, statistically, to change without compromising one of those processes (Duboule and Wilkins 1998; Otto 2004). In fact, we found that the higher the expression bias, the higher, on average, ω_α and ω are (as found also in (Duret and Mouchiroud 2000; Wright et al. 2004; Larracuente et al. 2008). The smaller the number of anatomical structures in which a gene is expressed, the higher ω. In spite of that, the anatomical structures showing the highest rates of adaptive substitution do not express genes with higher expression biases (Supplementary Figure 5C) or genes expressed in less anatomical structures than other anatomical structures (Supplementary Figure 5D). The same applies to the anatomical structures with low ω, they do not express genes that have low *Fop* (Supplementary Figure 5A) or are especially old (Supplementary Figure 5B)

compared to the genes expressed in other anatomical structures. In that respect, these results do not accommodate for easy genomic explanations but rather suggest that there may be some functional features of the “Garland cells/Plasmatocytes/Ring gland” and “Germ line” that have favored the accumulation of adaptive substitutions in the genes they express, at least compared with other parts of the anatomy, regardless of their pleiotropic effects.

Our results also indicate that the differences in ω_α and ω between anatomical structures are not related to differences in *Fop* or phylogenetic age. We do find, however, a negative relationship between *Fop* and ω_α and between *Fop* and phylogenetic age. A negative correlation between codon bias (*Fop*) and the rate of adaptive substitutions should be expected since for any given protein the codon changes that improve protein function would often be different from the codon changes associated with more efficient codon usage (Andolfatto 2007)shberg and Petrov 2008; Presnyak et al. 2015). The coding regions of older genes, in addition, have been molded by natural selection for longer times and, thus, are more likely to have reached and optimal codon usage (as observed). The reasons why phylogenetically more recent genes show higher ω_α and ω may be a bit more complex. On the one hand, we found that younger genes show higher expression biases and tend to be expressed in less anatomical structures than older genes. This suggests a scenario in which newly arising genes would start with very restricted expressions (both in anatomical space and developmental time) and have, thus, a low level of pleiotropy that would facilitate their evolution. On the other hand, older genes are more likely to be metabolic or housekeeping genes with essential functions that are unlikely to change in an adaptive way (Hastings 1996; Duret and Mouchiroud 2000; Zhang and Li 2004). Either way the fact is that the differences in rates of adaptive substitution between the sets of genes expressed in the different anatomical structures do not relate to differences in *Fop* or phylogenetic age. In other words, the anatomical structures expressing the set of genes with the highest ω_α and ω are not the ones expressing the genes with the lowest (or even especially low) *Fop* or expressing the youngest (or especially young) genes. Again, our results do not accommodate for easy genomic explanations such as *Fop* or phylogenetic age.

There are two anatomical structures that appear in our analysis as an exception to many of the trends identified. The salivary glands express relatively phylogenetically old genes that, in contrast to the rest of the ectoderm, exhibit rather high *Fop*. The high *Fop* may be explainable by both the old age of the genes expressed and the fact that many of the genes expressed in the salivary glands are well known to be expressed at very high levels (Andrew et al. 2000). Genes that are expressed at high levels are known to have, usually, rather high *Fop* because this allows for a faster and more

efficient translation (Gingold and Pilpel 2011; Quax et al. 2015), and we do in fact found this same result when plotting *Fop* against the levels of expression of genes (Supplementary Figure 7). We also found that while the endoderm as a germ layer seems to be under relaxed selection, the anatomical structure “Endoderm/Midgut” is under constraint. This apparent contradiction is explained by the fact that the germ layer analysis considers genes that are expressed exclusively in germ layer while anatomical structure analysis does not only consider genes that are expressed exclusively in an anatomical structure (because there is not enough of them). Out of the 303 genes that are exclusive of the endoderm, 206 (70%) are also annotated as “Maternal”. Maternal genes exhibit evidence of relaxed selection and, thus, explaining the relaxation observed in the “endoderm”.

The “Segmental/GAP” anatomical structure is also exceptional since it expresses the newest genes. These genes are expressed very early on before the germ layers form. Previous studies have shown that, in fact, there is substantial variation between diptera on which genes act early in development as segmental and gap genes (Wotton et al. 2015). Since these genes are all transcriptional factors it is not surprising that they are all relatively young (old genes tend to be metabolic genes involved in processes shared among distantly related groups).

There are a number of caveats to be considered in our results. First, it is not necessarily the case that the amount of adaptive amino acid substitutions in the set of genes expressed in an anatomical structure accurately reflects the amount of adaptive phenotypic change in that embryo part. Development is a complex process, with myriads of genetic and cell bio-mechanic interactions, that leads to a complex relationship between genetic variation and phenotypic variationerch 1982; Salazar-Ciudad 2006). It can be, for example, that some anatomical structures show only a small number of adaptive changes in the genes they express, and then not be detectable from our methods, but that those genetic changes have comparatively large effects on the phenotype. Second, we consider only changes in coding-regions while there is plenty of evidence of adaptation resulting from changes in regulatory regionsidson 2006). Third, variation in a gene can have an effect on anatomical structures where such gene is not expressed (e.g. extracellularly diffusible proteins or genes involved in the production mechanical forcesazar-Ciudad et al. 2003). Although all these caveats should be kept in mind there is no reason to expect that, a priori, the complexity of the genotype-phenotype map (or for that matter the amount of cis-regulation, signaling or mechanical forces) is dramatically different between anatomical structures. Then our comparison between anatomical structures is unlikely to be dramatically affected by these caveats.

Population genomics is concerned with genome variation, but natural selection acts upon the phenotype, not directly on the genotype, and the genomic dimension, albeit necessary, is not sufficient to account for a complete picture of organismal adaptation (Casillas and Barbadilla 2017). Trying to measure the action of natural selection in genes specifically expressed in different organs or across development can contribute to get a more systemic view of the causes and consequences of evolutionary and functional effects of genomic variation. This will also contribute towards aunified fitness-phenotype-genotype map in systems evolutionary biology (Casillas and Barbadilla 2017).

Material and methods

This section is divided into two parts. In the first one we describe how the expression data was obtained and processed to obtain a list of genes for each embryonic germ layer or anatomical structure. In the second part, we explain how genes expressed in these layers or structures were analyzed to estimate adaptation and other selective regimes as well as the statistical analyses performed.

Anatomical structures analysis

For the patterns of gene expression over the fly embryo's anatomy we used the BDGP database (Tomancak et al. 2007). This is a high-throughput database of mRNA expression spanning different embryonic stages. Each such stages actually includes a set of contiguous stages, thus, our stage 1 includes stage 1 to 3 of the fly development, stage 2 corresponds to stages 4 to 6, stage 3 to 7 and 8, stage 4 to 9 and 10, stage 5 includes stages 11 and 12 and stage 6 includes 13 to 16. This database has been the subject of previous statistical studies (Kumar et al. 2011; Salvador-Martínez and Salazar-Ciudad 2015) but not combined with populational genomic data as in here. Based on the expert analysis of whole-mount in situ RNA-hybridization images, the BDGP database contains, for each gene, the list of the embryonic anatomical structures in which it is expressed (http://insitu.fruitfly.org/insitu/html/downloads.html/). We removed genes with “no staining” as their unique term. We updated and validated those ID using FlyBase converting id tool obtaining 5,969 genes (http://flybase.org/static_pages/downloads/IDConv.html). Finally, we collapsed the original anatomical structure dataset into 18 different anatomical structures as described in Tomancak et al. 2007.

Germ layer analysis

To make a gene list for each germ we classified the anatomical structures by the germ layer to which they are derived from (e.g., dorsal epidermis is classified as ectoderm). A gene was assigned to a certain germ layer if it was expressed only in in anatomical structures belong to it (and in those of any other germ layer).

Genomic variables

Fop. The levels of codon bias, measured as *Fop* (Frequency of optimal codons) were estimated using CodonW (http://codonw.sourceforge.net/, Peden 1999). The *Fop* index

is the ratio of optimal codons to synonymous codons. Values range between 0 (where no optimal codons are used) and 1 (only optimal codons are used).

Expression bias and expression level. We have estimated expression bias, based on Yanai et al. 2005 formula (Yanai et al. 2005) see below), using modENCODE data (Graveley et al. 2011) (retrieved from FlyBase r6.06) from the stages "em0-2" to "em16-18" as the equivalent for stages 1 (1-3) and stage 6 (13-16):

$$\tau = \frac{\sum_{j=1}^{n} 1 - \left(\log S_j / \log S_{max}\right)}{n-1}$$

Where *S* is the logarithm of RPKM (Reads Per Kilobase per Million mapped reads) and *n* is the number of developmental stages. *τ* ranges from 0 to 1, with values close to 0 indicating broadly expressed genes and values close to 1 indicating genes with highly biased expression.

Phylogenetic age. We assigned a phylogenetic age to each gene using the phylostratigraphic maps of *D. melanogaster* from Drost 2014. These maps assign a phylogenetic age to each protein-coding gene in a species of interest (in this case *D. melanogaster*) based on the phylogenetic level at which orthologous for that gene are found (e.g., if a gene has orthologs at the level of eukaryota the phylogenetic age is older than if a gene has only orthologs among Drosophilids). With this method, each gene can be assigned a discrete age category, or phylostratum (PS), corresponding to hierarchically ordered phylogenetic nodes along the tree of life database (Drost et al. 2015). Although there is a concern regarding the accuracy of phylostratigraphy inferences, as they rely on BLAST searches, which show some limitations when sequences are highly diverged (Elhaik et al. 2005), Domazet-Lošo et al. 2017 have shown that this phylogenetic data is not biased and that BLAST is appropriate. In this work, they also show that the ectoderm is expressing evolutionary younger genes than in the endoderm and mesoderm. We downloaded the PS dataset on May 2015 (available in http://dx.doi.org/10.6084/m9.figshare.1244948/). As this dataset uses FlyBase protein IDs as identifiers, we used the R packages biomaRt (Durinck et al. 2005) version 2.22 and AnnotationDBI (Pages et al.) version 1.28.2 to convert them into FlyBase Gene IDs.

Population genomic analysis

In this section, we explain how we analyzed the genomic data in genes lists for each anatomical structure or layer to estimate adaptation and constraint.

Database

The population genomic data comes from 168 inbred lines of *D. melanogaster* sequenced in the Freeze 1.0 of the Drosophila Genetic Reference Panel (DGRP) project (Mackay et al. 2012) mapped to the release 5 of the Berkeley Drosophila Genome Project (http://www.fruitfly.org/sequence/release5genomic.shtml/). The DGRP population was created collecting gravid females from a single population of Raleigh, North Carolina (USA), followed by the full-sibling inbreeding approach during 20 generations to obtain full homozygous individuals. After this, the residual heterozygosis in the samples is expected to be 1.4% (inbreeding coefficient F = 0.986). The expected 1.4% of residual heterozygosis was true for 90% of the sequenced chromosome lines. DGRP lines showing high values of residual heterozygosity (>9%) were observed to be associated to large polymorphic inversions(Huang et al. 2014) and they were not included in the analyses (Huang et al. 2014). For computationally reasons, the program used for estimating the rate of adaptive substitution (DFE-alpha, see below) needs that all sites are sampled in the same number of individualsEyre-Walker and Keightley 2009). Hence, the original data of 168 lines set has been reduced to 128 isogenic lines by randomly sampling the polymorphisms at each site without replacement. Finally, residual heterozygous sites and sites with the lowest or no quality values were excluded from the analysis.

Estimation of natural selection on gene coding regions

We estimated natural selection on non-synonymous sites using the DFE-alpha software under a two-epoch demographic model (Eyre-Walker and Keightley 2009)Eyre-Walker and Keightley 2009). Coding exon annotations from *D. melanogaster* were retrieved from FlyBase (release 5.50, http://flybase.org/, last accessed March 2013). Genes 1:1 orthologs across *D. yakuba* - *D. melanogaster* were obtained from FlyBase (http://flybase.org/). We used *D. yakuba* as outgroup species because, aside from its high coverage (9.1x, Clark et al. 2007), the time elapsed since its divergence from *D. melanogaster* (7.4 MY, Tamura et al. 2004) is more suitable for estimating adaptation (Keightley and Eyre-Walker 2012). In closely related species (as is the case of *D. melanogaster* and *D. simulans*, which diverged 2.3 MY ago, Russo et al. 1995), the estimated rate of adaptive substitution can be biased due to (i) an erroneous attribution of polymorphism to divergence, (ii) ancestral polymorphism contributing to divergence and (iii) differences in the rate of fixation of neutral and adaptive mutations. These authors find that the adaptive rate estimated from closely related species (as in the case of *D. melanogaster* and *D. simulans*) may be underestimated by ~10% or more. For that, *D. yakuba* as outgroup gives a more reliable estimation of the adaptive rate than the closest *D. simulans* (Keightley and

Eyre-Walker 2012). A multiple genome alignment between the DGRP isogenic linesMackay et al. 2012) and the *D. yakuba* genomeClark et al. 2007) using the BDGP 5 coordinates was obtained from the publicly available database at http://popdrowser.uab.cat/Ràmia et al. 2012) (Ràmia et al. 2012). For each gene, we took all non-overlapping coding exons, independently of their inclusion levels. When two exons overlapped, the largest was chosen for subsequent analyses. Only exons without frameshifts, gaps or early stop codons were retained. In this way, we tried to avoid potential alignment errors that would inflate our mutation rate estimates and create an artefactual positive correlation between them. Exonic sequences were trimmed in order to contain only full codons. We calculated the number of substitutions and the folded site frequency spectrum (SFS, Ronen et al. 2013) for zero-fold degenerate sites, using an ad hoc Perl script. We used a custom-made Perl script to estimate the number of short intron substitutions and to compute the folded SFS. Jukes and Cantor correction for multiple hits was applied (Jukes and Cantor 1969)Jukes and Cantor 1969).

Several measures summarizing selection at the DNA level using data from polymorphism and divergence are inferred from the DFE-alpha method (Eyre-Walker and Keightley 2009). Briefly, this software uses a maximum-likelihood (ML) method based on polymorphism data to infer the distribution of fitness effects (DFE) of new mutations. It assumes two classes of sites in the genome: neutral sites (synonymous) and selected sites (nonsynonymous) and contrasts the site frequency spectrum (SFS) at these two classes. As a neutral reference, we used the 4-fold degenerate sites (and short intron sites for some cross-validating analysis, as it has been shown that evolve more neutral (Halligan and Keightley 2006). For our target sequence, we used 0-fold degenerate sites. Provided the SFS at both neutral and selected sites together with divergence data, allows DFE-alpha the calculation of α and ω_α. Furthermore, in our analysis we include another measure, ω_d, which represent the proportion of non-adaptive substitutions (slightly deleterious and neutral) relative to the neutral rate.

To estimate these selection measures, it is necessary to concatenate data from several genes because estimates from a single gene cannot be obtained due to the lack of segregating (or divergent) sites for some site classes. We only analyzed anatomical structures expressing a minimum of 150 genes to have enough statistical power.

Statistical analysis

Permutation test. To assess whether anatomical structures or germ layers undergo differential selection compared to other genes, a permutation test was applied. Permutation test are better suited for avoiding statistical Type I errors and are

considered a robust alternative to the Bonferroni correction dealing with dependent test (Sham and Purcell 2014). One of the main advantages of this method is that it can be applied to any statistic, and can incorporate distributional and dependence characteristics inherent to the data used, making it a robust test (Westfall and Young 1993). Importantly, using permutation tests, the null distribution is empirical, i.e., is obtained by calculating all possible, or a very large number of, values of the statistic under rearrangements of the labels on the observed data points (Berry et al. 2016). Therefore, in the case of our analysis, the null distribution of adaptive and constraint rates is different for the different analysis as each one is comprised of different number and combination of genes. Specifically, we first build a matrix where each column represents each anatomical structure or layer and each row represent a gene. The matrix is filled with 0 and 1, with 0 indicating no expression and 1 indicating expression of each gene in an anatomical structure or germ layer. To generate the expected null distribution, the gene ID labels in the matrix are re-shuffled at random. Each re-shuffle of the labels represents a new permuted dataset in which genes are distributed randomly between anatomical structures (or germ layers) while keeping constant the number of genes per anatomical structure (or germ layer) and the number of genes co-expressed between each anatomical structure (as in the original dataset). This allows us to infer the null distribution of the statistical output (α, ω, ω_a, ω_d) simultaneously for all the anatomical structures. This re-shuffling process was repeated 1,000 times to obtain the null distribution. A 2-tailed p-value was obtained by counting the number of replicates above or below the observed value in our analysis divided by the total number of replicates (1,000) and multiplying this value by 2 (Supplementary Figure 8).

T-test. We also calculated the significance of all the comparisons between germ layers and anatomical structures with a t-test. We compared the values (α, ω, ω_a, ω_d) measured in a given germ layer or anatomical structure against those measured in the set of genes that are not expressed in that given germ layer or anatomical structure. Each two groups were split in groups of 30 genes and were analyzed only if they had a minimum of 25 observation. The DFE-alpha measures are estimated for all these groups. The t-test is invalid for small samples from non-normal distributions, so we first check if the data follows a normal distribution using a Shapiro-Wilk test of normality. If the observations follow a non-normal distribution, we apply a non-parametric test (Wilcoxon test) instead of a t-test to perform the mean comparison. Finally, t-test (or Wilcoxon test) p-values were corrected by FDR.

Correlation analysis was performed in R (v3.3.2, R Core Team 2015) using the cor.test() function.

Figure legends

Figure 1. Preponderant selection on the genes expressed in each anatomical structure among stages. Stage 1. Relaxation on "Maternal". **Stage 2**. Selective constraint on "Ectoderm/Epidermis" and "Procephalic ectoderm/CNS" and positive selection on "Germ line". **Stage 3**. Selective constraint on the Intestinal tract ("Hindgut/Malpighian tubules", "Endoderm/Midgut") and positive selection on "Germ line". **Stage 4**. Selective constraint on "Mesoderm/Muscle", Intestinal tract ("Hindgut/Malpighian tubules", "Endoderm/Midgut"). **Stage 5**. Selective constraint on Intestinal tract ("Hindgut/Malpighian tubules", "Foregut", "Endoderm/Midgut"), "Procephalic ectoderm/CNS" and "Tracheal system". **Stage 6**. Selective constraint on "PNS", "Procephalic ectoderm/CNS", "Ectoderm/Epidermis", Intestinal tract ("Hindgut/Malpighian tubules", "Foregut", "Salivary glands", "Endoderm/Midgut"), and positive selection on "Head mesoderm/Circulatory system/Fat body" and "Germ line". Not shown: **Stage 3**. Selective constraint on "Ubiquitous" and "Ectoderm/Epidermis". **Stage 4**. Selective constraint on "Ubiquitous" and "Ectoderm/Epidermis". **Stage 5**. Selective constraint on "Ubiquitous", "Ectoderm/Epidermis", "Head mesoderm/Circulatory system/Fat Body". **Stage 6**. Selective constraint on "Ubiquitous", "Mesoderm/Muscle" and positive selection on "Garland/Plasmatocytes/Ring gland". See text for p-values and Supplementary Figure 2 for a schematic version of this figure. Since several anatomical structures under constraint overlap in the figure, some are represented in dark blue and some in light blue to facilitate visualization. Abbreviations: *amg*, anterior midgut rudiment; *pc*, pole cells; *hg*, hindgut; *pmg*, posterior midgut rudiment; *hms*, head mesoderm; *ms*, mesoderm; *mp*; Malpighian tubules; *fb*, fat body; *mg,* midgut; *go*, gonads; *sg*, salivary glands. Images modified from Hartenstein 1993 with permission.

Figure 2 Relationship between phylogenetic age and expression bias and *Fop*. A. Positive correlation between phylogenetic age and expression bias. B. Negative

correlation between phylogenetic age and *Fop*. Each dot represents the mean of each anatomical structure. It is represented the germ layer of origin of each anatomical structure. Blue: ectoderm origin, yellow: endoderm origin, green: mesoderm origin, red.

Figure 3. Relationship between spatial pleiotropy and the phylogenetic age, expression bias and *Fop*. The gene dataset was divided in eight groups depending on the number of anatomical structures in which they are expressed (1, 2, …, 7, 8 or more, see Supplementary Figure 4). Each group is obtained resampling with replacement 100 times the genes in each group. **A**. Negative correlation between the spatial pleiotropy and phylogenetic age. **B**. Negative correlation spatial pleiotropy and expression bias. **C**. Positive correlation between the spatial pleiotropy and the *Fop*.

Figure 4. Relationship between spatial pleiotropy and selective constraint, ω (A), relaxation, ω_d (B), and adaptation, ω_α (C). **A**. A negative correlation is found between ω and spatial pleiotropy. **B**. A negative correlation is found between ω_d and spatial pleiotropy. **C**. No correlation is found between ω_α and the gene groups. Each group is estimated by resampling with replacement 100 times the genes in each group.

Figures

Figure 1

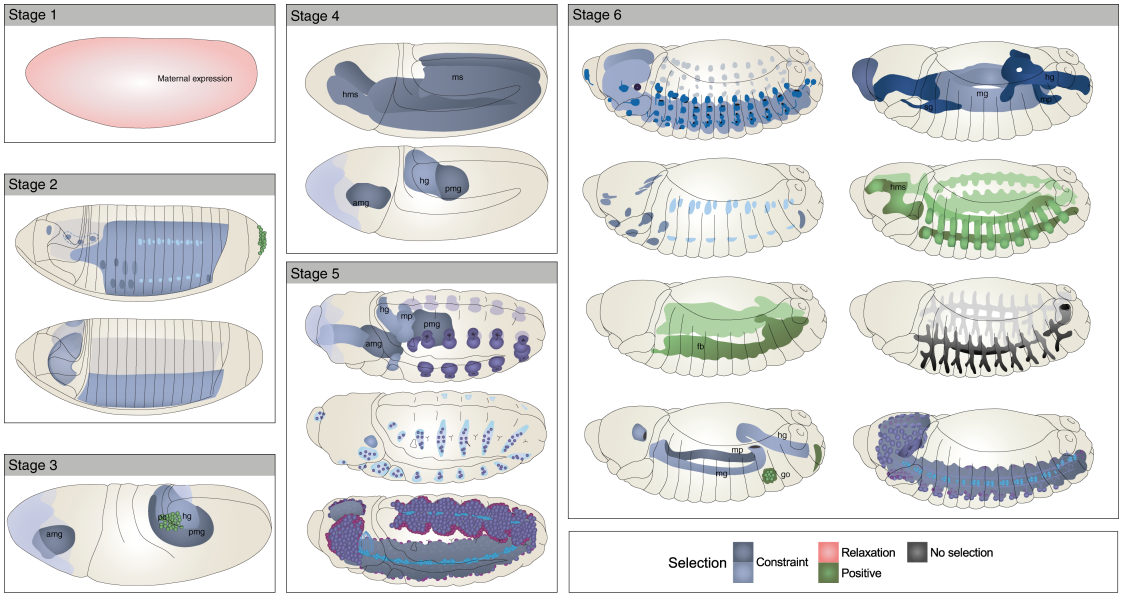


Figure 2

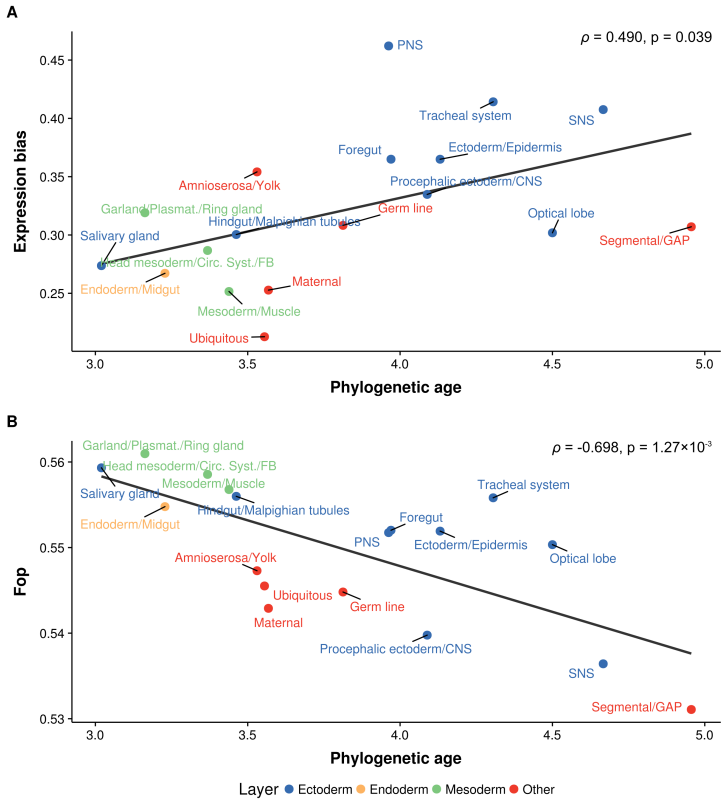


Figure 3

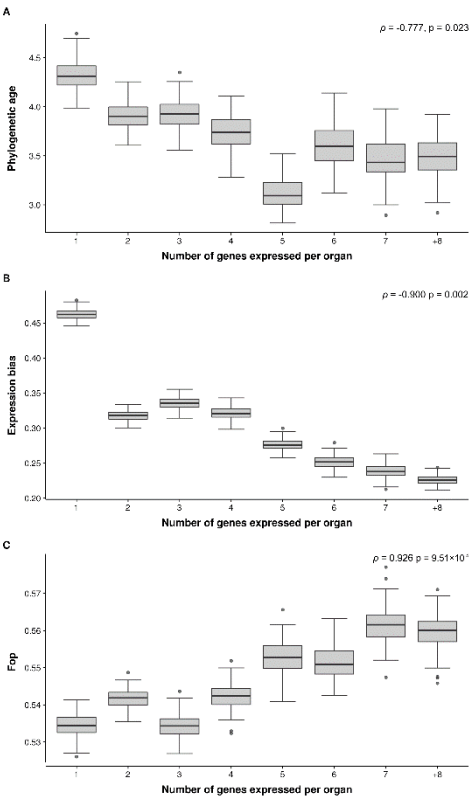
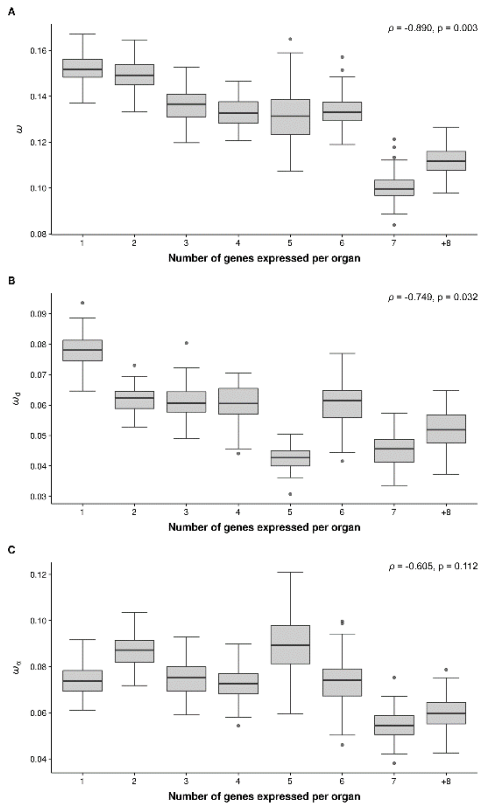


Figure 4



Acknowledgements

We thank Alfredo Ruiz, Sònia Casillas, Roland Zimm, Jukka Jernvall, Miquel Marin-Riera and Pascal Hagolani for useful comments. This research was funded by the Finnish Academy to IS-C and the Spanish Ministerio de Economía, Indústria y Competitividad Project BFU2013-42649-P to AB and the Generalitat de Catalunya grant 2014SGR1346 and FI-DGR 2015 to MCZ.

References

Alberch P. 1982. Evolution and Development. (Bonner JT, editor.). Berlin, Heidelberg: Springer Berlin Heidelberg

Andolfatto P. 2007. Hitchhiking effects of recurrent beneficial amino acid substitutions in the Drosophila melanogaster genome. Genome Res. 17:1755–1762.

Andrew DJ, Henderson KD, Seshaiah P. 2000. Salivary gland development in Drosophila melanogaster. Mech. Dev. 92:5–17.

Arbeitman MN, Furlong EEM, Imam F, Johnson E, Null BH, Baker BS, Krasnow MA, Scott MP, Davis RW, White KP. 2002. Gene expression during the life cycle of Drosophila melanogaster. Science 297:2270–2275.

Artieri CG, Haerty W, Singh RS. 2009. Ontogeny and phylogeny: molecular signatures of selection, constraint, and temporal pleiotropy in the development of Drosophila. BMC Biol. 7:42.

Assis R, Zhou Q, Bachtrog D. 2012. Sex-biased transcriptome evolution in Drosophila. Genome Biol. Evol. 4:1189–1200.

Bauer DuMont VL, Flores HA, Wright MH, Aquadro CF. 2007. Recurrent Positive Selection at Bgcn, a Key Determinant of Germ Line Differentiation, Does Not Appear to be Driven by Simple Coevolution with Its Partner Protein Bam. Mol. Biol. Evol. 24:182–191.

Berry KJ, Mielke, PW, Johnston JE. 2016. Permutation Statistical Methods. Cham: Springer International Publishing

Casillas S, Barbadilla A. 2017. Molecular Population Genetics. Genetics 205:1003–1035.

Christesen D, Yang YT, Somers J, Robin C, Sztal T, Batterham P, Perry T. 2016. Transcriptome Analysis of Drosophila melanogaster Third Instar Larval Ring Glands Points to Novel Functions and Uncovers a Cytochrome p450 Required for Development. G3 Genes, Genomes, Genet.

Civetta A, Rajakumar SA, Brouwers B, Bacik JP. 2005. Rapid Evolution and Gene-Specific Patterns of Selection for Three Genes of Spermatogenesis in Drosophila. Mol. Biol. Evol. 23:655–662.

Civetta A, Singh RS. 1995. High divergence of reproductive tract proteins and their association with postzygotic reproductive isolation in Drosophila melanogaster and Drosophila virilis group species. J. Mol. Evol. 41:1085–1095.

Clark AG, Eisen MB, Smith DR, Bergman CM, Oliver B, Markow TA, Kaufman TC, Kellis M, Gelbart W, Iyer VN, et al. 2007. Evolution of genes and genomes on the Drosophila phylogeny. Nature 450:203–218.

Davis JC, Brandman O, Petrov DA. 2005. Protein evolution in the context of Drosophila development. J. Mol. Evol. 60:774–785.

Domazet-Lošo T, Carvunis A-R, Albà MM, Šestak MS, Bakaric R, Neme R, Tautz D. 2017. No Evidence for Phylostratigraphic Bias Impacting Inferences on Patterns of Gene Emergence and Evolution. Mol. Biol. Evol. 34:843–856.

Drost H-G. 2014. A framework to perform phylotranscriptomics analyses for evolutionary developmental biology research.

Drost H-G, Gabel A, Grosse I, Quint M. 2015. Evidence for active maintenance of phylotranscriptomic hourglass patterns in animal and plant embryogenesis. Mol. Biol. Evol. 32:1221–1231.

Drummond DA, Bloom JD, Adami C, Wilke CO, Arnold FH. 2005. Why highly expressed proteins evolve slowly. Proc. Natl. Acad. Sci. U. S. A. 102:14338–14343.

Duboule D, Wilkins AS. 1998. The evolution of “bricolage”. Trends Genet. 14:54–59.

Duret L, Mouchiroud D. 2000. Determinants of Substitution Rates in Mammalian Genes: Expression Pattern Affects Selection Intensity but Not Mutation Rate. Mol. Biol. Evol. 17:68–70.

Durinck S, Moreau Y, Kasprzyk A, Davis S, De Moor B, Brazma A, Huber W. 2005. BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. Bioinformatics 21:3439–3440.

Early AM, Arguello JR, Cardoso-Moreira M, Gottipati S, Grenier JK, Clark AG. 2017. Survey of Global Genetic Diversity Within the Drosophila Immune System. Genetics 205.

Elhaik E, Sabath N, Graur D. 2005. The "Inverse Relationship Between Evolutionary Rate and Age of Mammalian Genes" Is an Artifact of Increased Genetic Distance with Rate of Evolution and Time of Divergence. Mol. Biol. Evol. 23:1–3.

Eyre-Walker A. 2002. Changing effective population size and the McDonald-Kreitman test. Genetics 162:2017–2024.

Eyre-Walker A, Keightley PD. 2009. Estimating the rate of adaptive molecular evolution in the presence of slightly deleterious mutations and population size change. Mol. Biol. Evol. 26:2097–2108.

Gingold H, Pilpel Y. 2011. Determinants of translation efficiency and accuracy. Mol. Syst. Biol. 7:481.

Gossmann TI, Song B-H, Windsor AJ, Mitchell-Olds T, Dixon CJ, Kapralov M V, Filatov DA, Eyre-Walker A. 2010. Genome wide analyses reveal little evidence for adaptive evolution in many plant species. Mol. Biol. Evol. 27:1822–1832.

Graveley BR, Brooks AN, Carlson JW, Duff MO, Landolin JM, Yang L, Artieri CG, van Baren MJ, Boley N, Booth BW, et al. 2011. The developmental transcriptome of Drosophila melanogaster. Nature 471:473–479.

Haerty W, Jagadeeshan S, Kulathinal RJ, Wong A, Ravi Ram K, Sirot LK, Levesque L, Artieri CG, Wolfner MF, Civetta A, et al. 2007. Evolution in the fast lane: rapidly evolving sex-related genes in Drosophila. Genetics 177:1321–1335.

Halligan DL, Keightley PD. 2006. Ubiquitous selective constraints in the Drosophila genome revealed by a genome-wide interspecies comparison. Genome Res. 16:875–884.

Hanada K, Shiu S-H, Li W-H. 2007. The Nonsynonymous/Synonymous Substitution Rate Ratio versus the Radical/Conservative Replacement Rate Ratio in the Evolution of Mammalian Genes. Mol. Biol. Evol. 24:2235–2241.

Hartenstein V. 1993. Atlas of Drosophila development. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

Hastings KE. 1996. Strong evolutionary conservation of broadly expressed protein isoforms in the troponin I gene family and other vertebrate gene families. J. Mol. Evol. 42:631–640.

Hereford J, Hansen TF, Houle D. 2004. Comparing strengths of directional selection: how strong is strong? Evolution 58:2133–2143.

Hershberg R, Petrov DA. 2008. Selection on codon bias. Annu. Rev. Genet. 42:287–

299.

Huang W, Massouras A, Inoue Y, Peiffer J, Ràmia M, Tarone AM, Turlapati L, Zichner T, Zhu D, Lyman RF, et al. 2014. Natural variation in genome architecture among 205 Drosophila melanogaster Genetic Reference Panel lines. Genome Res. 24:1193–1208.

Hudson RR, Kreitman M, Aguadé M. 1987. A test of neutral molecular evolution based on nucleotide data. Genetics 116:153–159.

Hurst LD. 2002. The Ka/Ks ratio: diagnosing the form of sequence evolution. Trends Genet. 18:486–487.

Jiggins FM, Kim KW. 2007. A screen for immunity genes evolving under positive selection in Drosophila. J. Evol. Biol. 20:965–970.

Jukes TH, Cantor CR. 1969. Evolution of protein molecules. In: Munro HN, editor. Mammalian Protein Metabolism. Academic Press, New York. p. 21–32.

Kalinka AT, Varga KM, Gerrard DT, Preibisch S, Corcoran DL, Jarrells J, Ohler U, Bergman CM, Tomancak P. 2010. Gene expression divergence recapitulates the developmental hourglass model. Nature 468:811–814.

Keightley PD, Eyre-Walker A. 2012. Estimating the rate of adaptive molecular evolution when the evolutionary divergence between species is small. J. Mol. Evol. 74:61–68.

Kimura M. 1957. Some Problems of Stochastic Processes in Genetics. Ann. Math. Stat. 28:882–901.

Kimura M. 1968. Evolutionary Rate at the Molecular Level. Nature 217:624–626.

Kumar S, Konikoff C, Van Emden B, Busick C, Davis KT, Ji S, Wu L-W, Ramos H, Brody T, Panchanathan S, et al. 2011. FlyExpress: visual mining of spatiotemporal patterns for genes and publications in Drosophila embryogenesis. Bioinformatics 27:3319–3320.

Larracuente AM, Sackton TB, Greenberg AJ, Wong A, Singh ND, Sturgill D, Zhang Y, Oliver B, Clark AG, Anthony J, et al. 2008. Evolution of protein-coding genes in Drosophila. Trends Genet. 24:114–123.

Lemos B, Bettencourt BR, Meiklejohn CD, Hartl DL. 2005. Evolution of proteins and gene expression levels are coupled in Drosophila and are independently associated with mRNA abundance, protein length, and number of protein-protein interactions. Mol. Biol. Evol. 22:1345–1354.

Mackay TFC, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, Casillas S, Han Y, Magwire MM, Cridland JM, et al. 2012. The Drosophila melanogaster Genetic Reference Panel. Nature 482:173–178.

Marais G, Domazet-Loso T, Tautz D, Charlesworth B. 2004. Correlated evolution of synonymous and nonsynonymous sites in Drosophila. J. Mol. Evol. 59:771–779.

McDonald JH, Kreitman M. 1991. Adaptive protein evolution at the Adh locus in Drosophila. Nature 351:652–654.

Meiklejohn CD, Parsch J, Ranz JM, Hartl DL. 2003. Rapid evolution of male-biased gene expression in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 100:9894–9899.

Mensch J, Serra F, Lavagnino NJ, Dopazo H, Hasson E. 2013. Positive selection in nucleoporins challenges constraints on early expressed genes in Drosophila development. Genome Biol. Evol. 5:2231–2241.

Obbard DJ, Welch JJ, Kim K-W, Jiggins FM. 2009. Quantifying adaptive evolution in the Drosophila immune system. PLoS Genet. 5:e1000698.

Otto SP. 2004. Two steps forward, one step back: the pleiotropic effects of favoured alleles. Proceedings. Biol. Sci. 271:705–714.

Pages H, Carlson M, Falcon S, Li N. AnnotationDbi: Annotation Database Interface.

Pal C, Papp B, Hurst LD, Pál C, Papp B, Hurst LD. 2001. Highly expressed genes in yeast evolve slowly. Genetics 158:927–931.

Peden JF. 1999. Analysis of codon usage.

Piasecka B, Lichocki P, Moretti S, Bergmann S, Robinson-Rechavi M. 2013. The hourglass and the early conservation models--co-existing patterns of developmental constraints in vertebrates. PLoS Genet. 9:e1003476.

Pröschel M, Zhang Z, Parsch J. 2006. Widespread adaptive evolution of Drosophila genes with sex-biased expression. Genetics 174:893–900.

Quax TEF, Claassens NJ, Söll D, van der Oost J. 2015. Codon Bias as a Means to Fine-Tune Gene Expression. Mol. Cell 59:149–161.

R Core Team. 2015. R: A Language and Environment for Statistical Computing.

Ràmia M, Librado P, Casillas S, Rozas J, Barbadilla A. 2012. PopDrowser: the Population Drosophila Browser. Bioinformatics 28:595–596.

Ratheesh A, Belyaeva V, Siekhaus DE. 2015. Drosophila immune cell migration and adhesion during embryonic development and larval immune responses. Curr. Opin. Cell Biol. 36:71–79.

Rocha EPC, Danchin A. 2004. An analysis of determinants of amino acids substitution rates in bacterial proteins. Mol. Biol. Evol. 21:108–116.

Ronen R, Udpa N, Halperin E, Bafna V. 2013. Learning natural selection from the site frequency spectrum. Genetics 195:181–193.

Roux J, Robinson-Rechavi M. 2008. Developmental constraints on vertebrate genome evolution. PLoS Genet. 4:e1000311.

Russo CA, Takezaki N, Nei M. 1995. Molecular phylogeny and divergence times of drosophilid species. Mol. Biol. Evol. 12:391–404.

Salathe M, Ackermann M, Bonhoeffer S, Salathé M, Ackermann M, Bonhoeffer S. 2006. The effect of multifunctionality on the rate of evolution in yeast. Mol. Biol. Evol. 23:721–722.

Salazar-Ciudad I. 2007. On the origins of morphological variation, canalization, robustness, and evolvability. Integr. Comp. Biol. 47:390–400.

Salvador-Martínez I, Salazar-Ciudad I. 2015. How complexity increases in development: An analysis of the spatial-temporal dynamics of 1218 genes in Drosophila melanogaster. Dev. Biol. 405:328–339.

Sawyer SA, Hartl DL. 1992. Population genetics of polymorphism and divergence. Genetics 132.

Schlenke TA, Begun DJ. 2003. Natural selection drives Drosophila immune system evolution. Genetics 164:1471–1480.

Sella G, Petrov D a, Przeworski M, Andolfatto P. 2009. Pervasive natural selection in the Drosophila genome? PLoS Genet. 5:e1000495–e1000495.

Sham PC, Purcell SM. 2014. Statistical power and significance testing in large-scale genetic studies. Nat. Rev. Genet. 15:335–346.

Sharp PM. 1991. Determinants of DNA sequence divergence between Escherichia coli and Salmonella typhimurium: codon usage, map position, and concerted evolution. J. Mol. Evol. 33:23–33.

Subramanian S, Kumar S. 2004. Gene expression intensity shapes evolutionary rates of the proteins encoded by the vertebrate genome. Genetics 168:373–381.

Tamura K, Subramanian S, Kumar S. 2004. Temporal patterns of fruit fly (Drosophila) evolution revealed by mutation clocks. Mol. Biol. Evol. 21:36–44.

Tomancak P, Berman BP, Beaton A, Weiszmann R, Kwan E, Hartenstein V, Celniker SE, Rubin GM. 2007. Global analysis of patterns of gene expression during Drosophila embryogenesis. Genome Biol. 8:R145.

Vitti JJ, Grossman SR, Sabeti PC. 2013. Detecting Natural Selection in Genomic Data. Annu. Rev. Genet. 47:97–120.

Westfall PH, Young SS. 1993. Resampling-Based Multiple Testing: Examples and Methods for p-Value Adjustment. Wiley, New York.

Wolf YI, Novichkov PS, Karev GP, Koonin E V, Lipman DJ. 2009. The universal distribution of evolutionary rates of genes and distinct characteristics of eukaryotic genes of different apparent ages. Proc. Natl. Acad. Sci. U. S. A. 106:7273–7280.

Wotton KR, Jiménez-Guri E, Crombach A, Cicin-Sain D, Jaeger J. 2015. High-resolution gene expression data from blastoderm embryos of the scuttle fly Megaselia abdita. Sci. data 2:150005.

Wright SI, Yau CBK, Looseley M, Meyers BC. 2004. Effects of gene expression on molecular evolution in Arabidopsis thaliana and Arabidopsis lyrata. Mol. Biol. Evol. 21:1719–1726.

Wyckoff GJ, Wang W, Wu CI. 2000. Rapid evolution of male reproductive genes in the descent of man. Nature 403:304–309.

Yanai I, Benjamin H, Shmoish M, Chalifa-Caspi V, Shklar M, Ophir R, Bar-Even A, Horn-Saban S, Safran M, Domany E, et al. 2005. Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. Bioinformatics 21:650–659.

Yang L, Wang Y, Zhang Z, He S. 2014. Comprehensive transcriptome analysis reveals accelerated genic evolution in a Tibet fish, Gymnodiptychus pachycheilus. Genome Biol. Evol. 7:251–261.

Yang W, Qi Y, Bi K, Fu J. 2012. Toward understanding the genetic basis of adaptation to high-elevation life in poikilothermic species: A comparative transcriptomic

analysis of two ranid frogs, Rana chensinensis and R. kukunoris. BMC Genomics 13:588.

Zhang G, Wang H, Shi J, Wang X, Zheng H, Wong GK-S, Clark T, Wang W, Wang J, Kang L. 2007. Identification and characterization of insect-specific proteins by genome data analysis. BMC Genomics 8:93.

Zhang L, Li W-H. 2004. Mammalian Housekeeping Genes Evolve More Slowly than Tissue-Specific Genes. Mol. Biol. Evol. 21:236–239.

Zhao L, Zhang N, Ma P-F, Liu Q, Li D-Z, Guo Z-H. 2013. Phylogenomic Analyses of Nuclear Genes Reveal the Evolutionary Relationships within the BEP Clade and the Evidence of Positive Selection in Poaceae. PLoS One 8:e64642.