The Genetic Architecture of Robustness for Flight Performance in *Drosophila*

Working title: Robustness of flight performance

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Author contributions

DMR and ANS conceived the idea and designed the experiment. ANS performed experiments and analyses. DMR and ANS wrote and revised the manuscript.

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Data accessibility statement

All phenotype data required to run the outlined analyses are available in Table S1 or using the DGRP2 webserver (<http://dgrp2.gnets.ncsu.edu/>). Supplemental tables and files are available at: <https://doi.org/10.7910/DVN/RSJNW5>.

Conflict of interest statement

The authors declare no conflict of interest.

ABSTRACT

A central challenge of quantitative genetics is partitioning phenotypic variation into genetic and non-genetic components. These non-genetic components are usually interpreted as environmental effects; however, variation between genetically identical individuals in a common environment can still exhibit phenotypic variation. A trait's resistance to variation is called robustness, though the genetics underlying it are poorly understood. Accordingly, we performed an association study on a previously studied, whole organism trait: flight performance. Using 197 of the Drosophila Genetic Reference Panel (DGRP) lines, we surveyed variation at the level of single nucleotide polymorphisms and whole genes using additive, marginal, and epistatic analyses that associated with robustness for flight performance. Many genes had developmental and neurodevelopmental annotations, and many more were identified from associations that differed between sexes. Additionally, many genes were pleiotropic, with several annotated for fitness-associated traits (e.g. gametogenesis and courtship). Our results corroborate a previous study for genetic modifiers of micro-environmental variation, and have sizable overlap with studies for modifiers of wing morphology and courtship behavior. These results point to an important and shared role for genetic modifiers of robustness of flight performance affecting development, neurodevelopment, and behavior.

Keywords: Drosophila Genetic Reference Panel (DGRP), genome wide association study (GWAS), quantitative genetics, flight performance, robustness, behavior

INTRODUCTION

Evolution acts on the genetic variation underlying phenotypic variation among individuals and populations. While many research programs focus on understanding genetic factors that contribute to phenotypic variation, fewer focus on non-genetic factors. The phenomenon of non-genetic (micro-environmental) variation describes the phenotypic variation that occurs in the absence of genetic variation, best studied in the genetically identical individuals. Non-genetic variation can arise from external (environmental) or internal (developmental) factors. Phenotypic variation across different environmental conditions (e.g. temperature) in genetically homogenous organisms is termed phenotypic plasticity. However, significant phenotypic variation can also arise among genetically homogeneous organisms in the absence of explicit environmental variation. Here, internal factors spur developmental noise in stochastic molecular processes (e.g. important transcripts or signals in very low abundance) that can result in varying levels of developmental stability (ALBAYRAK *et al.* 2016; SCHOR *et al.* 2017; KLINGENBERG 2019). The processes or ability for organisms to maintain a consistent phenotype in the presence of these perturbations is termed buffering, while the resulting phenotype is deemed robustness (KLINGENBERG 2019).

Developmental noise can affect an organism's developmental trajectory, which may impact the efficacy of natural selection by altering the association between genotype and phenotype. While it is difficult to directly observe developmental noise, deviations from an expected phenotype provide an adequate lens for study (MORGANTE *et al.* 2015;

VOGT 2015). An example of this is deviations in bilateral symmetry (fluctuating asymmetry) (VALEN 1962; SOTO *et al.* 2008), which are hypothesized to be negatively associated with fitness in the case of facial symmetry (QUINTO-SANCHEZ *et al.* 2018; LAJUS *et al.* 2019). Some genetic safeguards exist to buffer against developmental noise and maintain phenotypic robustness in the presence of these stressors. Chaperonins (HSP90) do so by maintaining a protein's structure during stressful times (RUTHERFORD AND LINDQUIST 1998; CHEN AND WAGNER 2012), as does the mitochondrial unfolded protein response in maintaining homeostasis and promoting longevity (PELLEGRINO *et al.* 2013; JOVAISAITE *et al.* 2014). In contrast, certain neurodevelopmental cell-cell adhesion molecules (e.g. DSCAMs, cadherins, and teneurins) leverage developmental noise to create more robust neural networks. In doing so, they drive repeatable non-genetic phenotypic variation in behavioral responses to serve as a bet hedging strategy (VOGT *et al.* 2008; AYROLES *et al.* 2015; HIESINGER AND HASSAN 2018; HONEGGER AND DE BIVORT 2018).

Genes that modulate a system's ability to resist developmental noise or a stressor are hypothesized evolutionary targets (WAGNER 2008; VOGT 2015; MENEZES *et al.* 2018) and subject to natural selection. And yet, these sources of non-genetic phenotypic variation are poorly understood. Previous studies have employed a Genome Wide Association Study (GWAS) framework on trait robustness, demonstrating the strategy's feasibility as they identify significant genetic modifiers (KAIN *et al.* 2012; AYROLES *et al.* 2015; MORGANTE *et al.* 2015; MENEZES *et al.* 2018; ROMAN *et al.* 2018). Similarly, we sought to elucidate these genetic factors by studying the robustness of flight performance in a

GWAS framework. We turned to the Drosophila Genetics Reference Panel (DGRP) lines, a collection of 205 genetically distinct and inbred lines of *D. melanogaster* that represent a snapshot of natural variation in a wild population (MACKAY *et al.* 2012; HUANG *et al.* 2014). Using a flight column to test flies' ability to react and respond to an abrupt drop (BENZER 1973; BABCOCK AND GANETZKY 2014), we tested 197 DGRP lines for their mean-normalized standard deviation (coefficient of variation) in flight performance. The coefficient of variation serves as a proxy for studying phenotypic robustness for genetically distinct groups comprised of genetically identical individuals. We identified significant additive, marginal, and epistatic variants, as well as whole gene effects on flight performance, across four sex-based phenotypes (males, females, and the average (sex-average) and difference (sex-difference) between sexes). Using defined insertional mutations in several candidate genes (*bru1*, *CadN*, *CG15236*, *CG32181/Adgf-A/Adgf-A2*, *CG3222*, *flippy/CG9766*, *CREG*, *Dscam4*, *flapper/CG11073*, *Form3*, *fry*, *Lasp/CG9692*, *Pde6*, *Snoo*), we successfully validated their gene roles affecting robustness of flight performance. These gene annotations suggest important roles for general and neurodevelopmental processes, and regulation of gene expression. Additionally, we found important roles for pleiotropic genes and fitness-associated genes, suggesting potential sources of developmental noise that may affect developmental stability. Together, these results contribute to a growing body of literature surrounding the genetics of robustness and corroborate previous screens for micro-environmental plasticity (MORGANTE *et al.* 2015), wing morphology (PITCHERS *et al.* 2019), and courtship behaviors (TURNER *et al.* 2013; GAERTNER *et al.* 2015).

METHODS

Drosophila Stocks and Husbandry

197 *Drosophila* Genetic Reference Panel (DGRP) lines (HUANG *et al.* 2014) and 24
stocks used in the validation experiment were obtained from Bloomington *Drosophila*
Stock Center (Table S1; <https://bdsc.indiana.edu/>). Flies were grown on a standard
cornmeal media (MOSSMAN *et al.* 2016) at 25° under a 12h:12h light-dark cycle. Two to
three days post-eclosion, they were sorted by sex under light CO₂ anesthesia and given
five days to recover before assaying flight performance.

Flight performance assay

We tested approximately 100 flies of each sex from 197 DGRP genotypes (Table S1)
using a refined protocol (BABCOCK AND GANETZKY 2014) for measuring flight
performance (BENZER 1973). For each sex-genotype combination, groups of 20 flies in
five glass vials were knocked down, uncorked, and rapidly inverted down a 25 cm
chute. The vials traveled until they reached a stop, at which point flies were ejected into
a 100 cm long by 13.5 cm wide tube. Freefalling flies instinctively attempt to right
themselves and land. A transparent acrylic sheet coated in TangleTrap adhesive lined
the inside of the tube and immobilized flies at their respective landing height. The sheet,
was removed, pinned to a white poster board, and photographed using a Raspberry Pi
(model 3 B+) and PiCamera (V2). The positional coordinates were extracted using
ImageJ/FIJI's 'Find Maxima' feature with options for a light background and noise
tolerance of 30 (SCHINDELIN *et al.* 2012). The distributions of landing heights for each

sex-genotype combination were used to calculate the mean and standard deviation. The coefficient of variation, or the mean-normalized standard deviation, was used as the final phenotype value to represent robustness.

Genome wide association mapping

Robustness phenotypes (Table S2) were submitted to the DGRP2 webserver for the additive association analysis (<http://dgrp2.gnets.ncsu.edu/>) (MACKAY *et al.* 2012; HUANG *et al.* 2014), which returned association results for four sex-based phenotypes: males, females, average between sexes (sex-average) and difference between sexes (sex-difference). We analyzed 1,901,174 common variants (minor allele frequency ≥ 0.05) using a mixed effect model to account for Wolbachia infection status and presence of five major inversions. Since certain inversions covaried with the robustness phenotype (Table S3), only significance scores from a linear mixed model accounting for Wolbachia status and the presence of five major inversions were considered.

Validating candidate genes

Candidate genes (Table S1B) were selected if they were identified from variants identified in the sex-average additive variant screen for mean landing height and if there were publicly available lines containing a *Minos* enhancer trap (*Mi{ET1}*) mutational insertion (METAXAKIS *et al.* 2005) generated by the Drosophila Gene Disruption Project (BELLEN *et al.* 2011). Experimental and control lines were derived from common isoparental crosses for each candidate gene stock backcrossed for five generations to the respective w^{1118} or y^1w^{67c23} background. Isoparental crosses between the resulting

heterozygous offspring were partitioned for absence (control line) or presence (experimental line) of the *Mi{ET1}* construct. Experimental lines were verified for homozygosity if all progeny contained the insertion after several rounds of culturing. Validations were conducting in the flight performance assay described above. The distributions in landing heights were assessed for significance if they passed a $P \leq 0.05$ significance threshold in a Kolmogorov-Smirnov test comparing control and mutant genotypes.

Calculating gene-score significance

Gene-level significance scores (gene-score) were determined using PEGASUS_flies (SPIERER *et al.* 2020), a Drosophila-optimized method for the human-based platform Precise, Efficient Gene Association Score Using SNPs (PEGASUS) (NAKKA *et al.* 2016). This analysis calculates gene-scores for each gene as a test of whether the distribution of additive variants within a gene (accounting for linkage disequilibrium) deviates from a null chi-squared distribution. Variants from the additive association screen were considered and mapped onto gene annotations and linkage disequilibrium files available with the PEGASUS_flies package—derived initially from the DGRP2 webserver.

Screening for epistatic interactions

Marginal variants, corresponding with variants more likely to interact with other variants, were identified using MArginal ePIstasis Test (MAPIT) (CRAWFORD *et al.* 2017). This approach uses a Bayesian framework to test the marginal effect of each variant against a focal phenotype. MAPIT requires a complete genotype-phenotype matrix so the

DGRP genome was imputed for missing variants using BEAGLE 4.1 (BROWNING AND BROWNING 2007; BROWNING AND BROWNING 2016) and filtered for $MAF \geq 0.05$ using VCFtools (v.0.1.16) (DANECEK *et al.* 2011).

MAPIT was run using the 'Davies' method on the DGRP2 webserver's adjusted phenotype scores, 1,952,233 imputed and filtered variants, and relatedness and covariate status files available on the DGRP2 webserver (<http://dgrp2.gnets.ncsu.edu/data.html>). Marginal effect P-values (data available File S8) for each sex-based phenotype were filtered for a Bonferroni threshold ($P < 2.56e-8$) and served as a focused subset for targeted pairwise epistasis testing against the unimputed variants ($n = 1,901,174$). Epistatic interactions were calculated using the '–epistasis' test in a '–set-by-all' framework in PLINK (v.1.90) (PURCELL *et al.* 2007). Significant epistatic interactions were considered if they passed a Bonferroni threshold: $0.05 / (n \times 1901174 \text{ variants})$, where 'n' represents the number of significant marginal variants tested in a sex-specific subset (Table 3).

Annotating FBgn and orthologs

FB5.57 annotations for FlyBase gene numbers (FBgn) were converted to FB_2020_01 annotations using the FlyBase tool 'Upload/Convert IDs' (THURMOND *et al.* 2019). Updated FBgn (Dmel) were mapped to human orthologs (Hsap) using the Drosophila RNAi Stock Center (DRSC) Integrative Ortholog Prediction Tool (DIOPT)(Hu *et al.* 2011) tool, with the additional filtering parameter: "Return only best match when there is more than one match per input gene or protein." Annotations for various genes without a

citation were done so with auto-generated summaries and unreferenced descriptors of gene functions, expression profiles, and orthologs from FlyBase (GRUMBLING *et al.* 2006; DOS SANTOS *et al.* 2015). These descriptors were compiled from data supplied by the Gene Ontology Consortium (ASHBURNER *et al.* 2000; CARBON *et al.* 2019), the Berkeley Drosophila Genome Project (FRISE *et al.* 2010), FlyAtlas (CHINTAPALLI *et al.* 2007), The Alliance of Genome Resources Consortium (CONSORTIUM 2020), modENCODE (DOS SANTOS *et al.* 2015), DRSC Integrative Ortholog Prediction Tool (DIOPT) (HU *et al.* 2011), and Phylogenetic Annotation and INference Tool (PAINT) (GAUDET *et al.* 2011).

Data availability

All phenotype data required to run the outlined analyses are available in Table S2 or using the DGRP2 webserver (<http://dgrp2.gnets.ncsu.edu/>). All supporting information is available at: <https://doi.org/10.7910/DVN/RSJNW5>.

RESULTS and DISCUSSION

We sought to identify the genetic modifiers of robustness in a whole organism phenotype: flight performance. Using the Drosophila Genetic Reference Panel (DGRP) lines, we identified several additive, marginal, and epistatic variants, as well as whole genes that associate with genotype robustness in response to a flight challenge. In the sections that follow we describe the variant-based, gene-based, and epistatic analyses in turn.

Variation in flight performance across the DGRP

We screened 197 DGRP lines (Table S1) for their flight ability in response to an abrupt drop (Figure 1A). Qualitative observations made in a previous study of strong, intermediate, and weak genotypes in the flight assay suggests stronger genotypes react faster and respond more effectively than weaker ones (SPIERER *et al.* 2020). The mean and standard deviation in landing height were calculated for each sex-genotype combination, along with the mean-normalized standard deviation (coefficient of variation). The coefficient of variation served as our metric for robustness (Figure S1; Table S3). Genotypes with a lower coefficient of variation (more consistent) are more robust for flight performance (KLINGENBERG 2019). On average, flight performance was more robust in males than females (males: 0.17 A.U. $\pm$ 0.055 SD vs. females: 0.22 A.U. $\pm$ 0.075 SD; Figures 1B and S2). There was a significant relationship in robustness between sexes ($R = 0.55$; $P < 1E-16$; Figure 1C), suggesting the genetic architecture of

robustness in flight performance is similar between the sexes. However, the magnitude of the regression coefficient suggests robustness is somewhat sexual dimorphic.

We tested our phenotype in each sex against those publicly available on the DGRP2 webserver to determine whether robustness of flight performance was a unique trait. Indeed, we found no significant relationship after applying a multiple testing correction ($P \leq 1.85E-3$; Table S3), suggesting it is unique. Accordingly, we took four distinct approaches to find significant associations from different facets of the genetic architecture (Table 1).

Several variants of large effect associate with robustness in flight performance

We performed a Genome Wide Association Study (GWAS) to calculate the significance of variants' additive effects, and subsequently whole gene significance scores. We analyzed the effects of 1,901,174 common variants ($MAF \geq 0.05$) across for four sex-based phenotypes (males, females, the sex-average, and sex-difference; Figures 1D and S3-5). Two of the major inversions covaried with our phenotype scores (Table S4), so we used a mixed model to account for Wolbachia infection status, presence of inversions, and polygenic relatedness.

We performed a traditional, additive variant screen using the DGRP2 webserver pipeline. Eight variants in the male, female, or sex-average analyses passed a strict Bonferroni threshold ($P \leq 2.63E-8$; Tables 2-4; Table S5). Three variants (2R_17433667_SNP, 3R_4379159_SNP, 3R_9684126_SNP) were also significant for

the mean landing height in flight performance (SPIERER *et al.* 2020) and mapped to *Epidermal Growth Factor Receptor (Egfr*, human homolog *EGFR*), *Odorant receptor 85d (Or85d)* and an intergenic region on chromosome 3R, respectively. *Egfr* is a pleiotropic, tyrosine kinase receptor involved in several developmental and homeostatic processes, as well as playing a known source of natural variants that modify wing shape and affect flight performance (PAUL *et al.* 2013; PITCHERS *et al.* 2019). *Or85d* is an odorant receptor expressed on the antennae and maxillary palp (COUTO *et al.* 2005). The significant variant in *Or85d* coded for a non-synonymous SNP (C277Y) at highly conserved site (Figure S6) (SIEPEL *et al.* 2005; SIEPEL AND HAUSSLER 2005), though follow up analysis with the PROVEAN webtool (CHOI AND CHAN 2015) suggests this mutation is neutral (scored -2.312 with -2.5 as deleterious). Finally, the intergenic region lacked any embryonic transcription factor binding site (TFBS) annotations (MOSES *et al.* 2006; LI *et al.* 2008; MACARTHUR *et al.* 2009; THOMAS *et al.* 2011), suggesting it may interact with transcription factors or epigenetic factors later during development or homeostasis.

The remaining Bonferroni additive variants mapped to genes that were also identified from additive Bonferroni variants in the mean flight performance screen (*Dscam4* and *CG11073/flapper*) or were otherwise strongly significant (*Snoo*). These three genes have known or hypothesized roles in developing robust neural circuits (QUIJANO *et al.* 2010; TADROS *et al.* 2016; SPIERER *et al.* 2020). Their identification in mean and robustness of flight performance screens suggests they have a dual role in affecting a genotype's ability and variability in flight performance.

299

300 Applying a traditional DGRP association threshold ($P \leq 1E-5$), we observed a trend in
 301 our data where neural and pleiotropic genes play an important role in the genetic
 302 architecture of robustness of flight performance. We identified 163 unique, significant
 303 variants (Table S5), 18 of which mapped to coding regions (Table 4). These coding
 304 variants include a novel transcriptional start site in a gene (*CG43707*) affecting muscle
 305 architecture and flight performance (SCHNORRER *et al.* 2010) and six non-synonymous
 306 SNPs that each mapped to unique genes (*CG12517*, *CG13794*, *CG34215*, *Or85d*, *Spn*,
 307 *Tif-1A*). Some of these affect neural phenotypes, like *CG13794*, a neurotransmitter
 308 (COUTO *et al.* 2005; ROMERO-CALDERON *et al.* 2007), while others are pleiotropic and
 309 affect multiple traits. *CG12517* and *Tif-1A* are involved in the stress response of the fat
 310 body and insulin-based metabolism, respectively, and both are involved in development
 311 of the germline (YATSU *et al.* 2008; TOOTLE *et al.* 2011; TSUZUKI *et al.* 2012; GHOSH *et al.*
 312 2014). *Spn* (*Spinophilin*; human homolog *PPP1R9A*), a pre-synaptic regulator of
 313 neurons (MUHAMMAD *et al.* 2015), affects flight performance (SCHNORRER *et al.* 2010),
 314 male aggression (EDWARDS *et al.* 2009), odor response (SAMBANDAN *et al.* 2006), and is
 315 also found in sperm (WASBROUGH *et al.* 2010).

316

317 Most variants mapped to complex traits with the DGRP are enriched for intergenic and
 318 non-coding regions (Table 4) and have presumably regulatory roles (MACKAY *et al.*
 319 2012; MACKAY AND HUANG 2018). Non-coding variants mapped to genes with important
 320 roles in development and function of the nervous system, wing morphology, muscle
 321 function, and regulation of gene expression. They also play important roles in

gametogenesis or determination of sex identity, establishing a possible connection between genotype and the sexually dimorphic phenotype we measured. A table of all genes found in the current study is available as a master lookup table (File S1). The identification of these genes and their respective annotations may hint at additional genetic sources of developmental stabilization.

Functional validation of candidate genes supports a role for neurodevelopment affecting robustness of flight performance

We functionally validated several genes' roles in affecting robustness of flight performance. Using insertional mutants in the candidate genes identified from the mean landing height screen, we applied a Kolmogorov-Smirnov test between insert and backcross-control genotypes (see methods) to test for differences in the distribution of landing heights. We validated 11 single genes (*bru1*, *CadN*, *flippy* (CG9766), *CG15236*, *CREG*, *Dscam4*, *flapper* (CG11073), *form3*, *fry*, *Pde6*, and *Snoo*), and two constructs that fell in multiple genes (*Adgf-A/Adgf-A2/CG32181* and *CG9692/Lasp*) (Figures 2 and S7; Table S6). These genes are putative sources of genetic variation in maintaining developmental stability. They were also validated in the mean flight performance screen (SPIERER *et al.* 2020), suggesting these genes likely play dual roles modifying the ability and variability of flight performance.

Analyses of whole-gene effects identifies distinct factors affecting robustness

The additive variant screen takes a conventional minSNP approach, deeming a gene significant if its most significant variant passes a significance threshold. However, this

approach is biased toward longer genes and does not account for linkage between sites. To counteract these biases, we employed PEGASUS_flies (SPIERER *et al.* 2020), a *Drosophila* version of the human-focused PEGASUS platform (NAKKA *et al.* 2016). This method takes a gene-specific approach; assessing a whole gene's significance by testing the distribution of variants within a gene against a null chi-squared distribution of SNP *P*-values. Thus, it can detect significant genes of moderate effect, as well as genes that may be missed in a minSNP approach.

Using PEGASUS_flies, we identified 45 unique genes (Table S7) across all four sex-based phenotypes that passed a Bonferroni threshold ($P \leq 3.43E-6$; Figures 3A and S8). Two were present in the additive screen (*nmo* and *Sdc*), along with 27 other genes with annotations for neurodevelopment and function (*ana3*, *barc*, *Br140*, *caps*, *CG5921*, *CG5937*, *CG12163*, *CG44774*, *Crz*, *ct*, *ctrip*, *Dop2R*, *Dys*, *ena*, *ham*, *Nckx30C*, *Oct-TyR*, *olf186-F*, *PsGEF*, *Ptp4E*, *rad*, *rodgi*, *row*, *tou*, *TTLL5*, *tutl*, *wde*). Some genes also affected muscle, chaete, or general development (*caps*, *CG5937*, *CG31635*, *CG32521*, *CG3277*, *CG43333*), while others facilitated gametogenesis or promoted reproductive success (*ana3*, *CG1632*, *CG5937*, *CG12163*, *CG44774*, *CHES-1-like*, *Crz*, *ct*, *Dop2R*, *Dys*, *ena*, *Gbs-70E*, *PsGEF*, *tou*, *wde*). These results largely corroborate annotations from the additive search and expanded the number of genes that associate with robustness in flight performance.

Association of marginal and epistatic variants with robustness in flight performance

Epistatic, or pairwise, interactions play an outsized role as context-specific effectors in complex traits (HUANG *et al.* 2012). Traditional epistasis analyses face large computational and statistical hurdles, so we turned to MArginal ePIstasis Test (MAPIT) to focus the search for interactions (CRAWFORD *et al.* 2017). MAPIT is a Bayesian approach that identifies marginal variants representing genetic hubs. Using this subset of hub variants, we performed a set-by-all epistasis search against all other variants. We identified 104 significant marginal variants in 66 genes and several intergenic regions that exceeded a Bonferroni threshold ($P \leq 2.56E-8$) across all sex-based phenotypes (Figures 3B and S9; Table S8). Most of these variants mapped to non-coding regions, suggesting an important regulatory role among marginal variants (MACKAY AND HUANG 2018). Among the 19 the significant marginal variants with epistatic interactions (discussed below), only seven mapped to genes (*Bx*, *CG9313*, *CG15651*, *CG9171*, *PKC- δ /Pkcdelta*, *jvl*, *ush*).

In total, there were 6313 significant epistatic interactions that mapped to 1081 genes (Table 3). Interestingly, several of the marginal genes (containing marginal variants) had epistatic interactions with other marginal genes (marginal-marginal epistatic interactions; Figure 4A). However, these interactions were typically between a marginal and non-marginal variant, rather than between two marginal variants. This finding suggests a highly interconnected genetic architecture. Broadly, epistatic interactions were enriched for neurodevelopment and general development, though there were too many significant interactions to comprehensively describe in text (Table S9). Instead,

we will focus on the marginal variants that mapped to genes and some of their noteworthy epistatic interactions.

Many marginal variants in female and sex-average epistasis analyses map to pleiotropic genes

While the male marginal variant mapped to an intergenic region, there were several marginal variants in the female and sex-average analyses that mapped to genes, especially pleiotropic genes. Among these were *Beadex* (*Bx*; human homolog *LMO1*), *CG9171* (human homolog *B4GAT1*), *CG15651* (human homolog *FKRP*), *CG9313*, and *javelin-like* (*jvl*). The corresponding and significant epistatic interactions, like these marginal genes, have annotations for wing morphology, muscle development, neural circuit assembly and neuronal function, and interestingly, sex-related behaviors and sex-specific tissues (Table S8). For more information on marginal gene functions, please see "Many marginal variants in female and sex-average epistasis analyses map to pleiotropic genes, continued" in the Supplemental Results.

Epistatic interactions associating with the sex-difference phenotype

As noted previously, there was relationship between male and female robustness scores ($R = 0.55$) that suggests a genetic basis for sexual dimorphism. We found a signature of this dimorphism in the sex-difference epistasis search, which had four times as many epistatic interactions as the next closest sex-based phenotype (females).

Protein Kinase C-δ (*PKC-δ* or *Pkcd*; human homologs *PRKCD* and *PRKCQ*) drove this trend and accounted for over half (3211 of 6313) of all epistatic interactions in our study. Some of these interactions were with variants in other marginal genes (Figure 4A), suggesting a more central and interconnected role within the genetic network. *PKC-δ* is a member of the Protein Kinase C family and is known to modulate flies ability to learn from their environment, especially during flight (COLOMB AND BREMBS 2016; GETAHUN *et al.* 2016; GOROSTIZA *et al.* 2016). Flies inability to learn from proprioceptive cues corresponds increased variation in their flight path (HESSELBERG AND LEHMANN 2009; LEHMANN AND BARTUSSEK 2017). Thus, *PKC-δ* is a noteworthy candidate gene with a previously validated effect on flight robustness.

Another marginal variant from the sex-difference epistasis screen was the developmental transcription factor *u-shaped* (*ush*; human homolog *ZFPM1*), which mediates neurodevelopment and thorax development (FROMENTAL-RAMAIN *et al.* 2010). It regulates *scute* (*sc*), with roles in the sex-determination pathway (WRISCHNIK *et al.* 2003), and the SC-A (*scute* and *achaete*) complex, which contributes to development of mechanosensory chaete and sensory organs on the wing (SKEATH AND CARROLL 1991; CUBADDA *et al.* 1997). Accordingly, *ush* is similarly a pleiotropic gene that acts as a potential source of developmental noise and stability affecting robustness of flight performance. For more examples and information of epistatic interactions between *ush* and other genes, see "Epistatic interactions associating with the sex-difference phenotype, continued" in the Supplemental Results.

Bet hedging as a modifier of robustness

Bet hedging is an evolutionary strategy for increasing phenotypic variation in the presence of genetic homogeneity (HIESINGER AND HASSAN 2018; HONEGGER AND DE BIVORT 2018). We identified putative bet hedging genes involved with neurodevelopment, such as: Down Syndrome Cell Adhesion Molecules (DSCAM; *Dscam3* and *Dscam4*), cadherins (*Cad87A*, *CadN*, *CadN2*), and teneurin (*Ten-a*) family genes. These genes were also significant interactors with *PKC-δ*. They contribute to differential wiring of the peripheral and central nervous systems through dendritic self-avoidance (HONG *et al.* 2012; LI *et al.* 2020) in the brain and sensory organs of the wing (KISE AND SCHMUCKER 2013; NAGAI AND MIZUNO 2014). Interestingly, *Ten-a* was previously identified and validated in a screen for individuality in locomotor handedness (AYROLES *et al.* 2015) and we validated *CadN* and *Dscam4* in the present study. Together, these genes and their roles in differential circuit assembly serve as validated modulators of robustness.

Flight and courtship share morphological structures and genetic modifiers

Interestingly, we identified several pleiotropic genes with dual roles in robustness of flight performance and courtship and fitness-associated traits. One such gene from the marginal screen was *factor of interpulse interval (fipi)*, a marginal variant with no significant epistatic interactors. This gene regulates the intervals of courtship song (FEDOTOV *et al.* 2018) and was previously identified in an independent screen for micro-environmental variation (MORGANTE *et al.* 2015). Other courtship-related studies with the DGRP shared genes with robustness in flight performance (*CG1358* and *Dif*) (TURNER

et al. 2013) and (*bru-3*, *CG13024*, *CG42784*, *Fur1*, *shot*, *SKIP*, *Ubx*, *wuc*) (GAERTNER *et al.* 2015). We identified several other genes with annotations for sex determination, courtship behavior, and sex-specific neural patterning (*Alh*, *bab1*, *Btk29A*, *CASK*, *chinmo*, *dnc*, *dysb*, *fru*, *gom*, *lov*, *Mip*, *Nrg*, *Sh*, *Tbh*), as well as many genes with dual roles in gametogenesis (previously listed).

The link between pleiotropic genes association with both fitness-associated traits and robustness of flight performance may lie in the development of common structures. In flight, well-structured wings are important for generating lift (MARCUS 2001) and chaete are important for proprioception (FURMAN AND BUKHARINA 2008; QUIJANO *et al.* 2010). In courtship, wings are used for visual flagging and courtship song (SADAF *et al.* 2015), while chaete act as chemosensors for pheromones (THISTLE *et al.* 2012; PAVLOU AND GOODWIN 2013) and mechanosensors in courtship. Additionally, the neural networks innervating chaete and connecting them to the central nervous system (CNS) are differentially patterned by cell-cell adhesion molecules (e.g. DSCAMS, cadherins, and teneurins) (HONG *et al.* 2012). We successfully validated *Dscam4* and *CadN*, two such neural patterning genes with important implications in differential detection of pheromones and courtship behaviors (YU *et al.* 2010; PAVLOU AND GOODWIN 2013), and flight performance (SPIERER *et al.* 2020), lending support to our hypothesis.

These pleiotropic genes may be associated with robustness as a result of an evolutionary tug-of-war caused by intralocus sexual dimorphism or conflict. In other words, what is beneficial for one sex may be neutral or disadvantageous for the other.

This phenomenon is observed in insects in the context of locomotor performance, courtship behavior, and fitness (BERGER *et al.* 2014; BERGER *et al.* 2016). In studies where male flies were allowed to genetically “win” the sex conflict and evolve, males increased locomotor activity, fitness, and variation in wing morphology, while females saw a reduction in locomotor activity and fitness (LONG AND RICE 2007; ABBOTT *et al.* 2010). Variation in wing morphology is noteworthy because it is hypothesized to be under stabilizing selection (MUNOZ-MUNOZ *et al.* 2016; SZTEPANACZ *et al.* 2017). It is also sensitive to factors that buffer against developmental noise and serves as a strong proxy for developmental stability (SOTO *et al.* 2008; KLINGENBERG 2019). Therefore, flies with less within- and between-individual variation in wing morphology are thought to have better buffered genetic networks against developmental noise and stochasticity.

Genetic architecture of robustness is comprised of different types of modifiers

Each analysis we conducted sheds light on different areas of the genetic architecture (Table 1). The additive variant analysis identified single variants with larger effects on the phenotype, while the whole gene analysis identified genes of moderate effect based on the distribution of additive variants in a gene. The marginal variant analysis identified variants more likely to interact with other variants, while the epistasis analysis identified those respective interactions. Of the variant-based analyses, all additive variants were exclusive, though the marginal variants and epistatic interactions had some overlap (Figure 4B). When mapping these variants to genes, all analyses identified genes that were shared with at least two other analyses (Figure 4C). This result suggests that genes contain different types of variants that affect separate facets of the genetic

architecture. For a complete list of all genes identified in this study and which analysis they were present in, see File S1.

Overlap between robustness and other DGRP studies

When comparing screens for variants associating with overall flight performance (mean) against robustness for flight performance (coefficient of variation), we consistently identified approximately 15-20% overlap between variants and their mapped genes (Figure 4D-H; Table S10) with the exception of the whole gene analysis (no overlap; Figure 4I). These results suggest that while certain main features of the genetic architecture are shared, the traits have largely separate genetic architectures.

Similarly, we found commonalities between robustness in flight performance and other DGRP studies conducted beyond the flight phenotype. A micro-environmental plasticity screen for startle response, resistance to starvation, and chill coma recovery shared 37 genes with robustness of flight performance (*Bsg25D*, *CARPB*, *CG17716*, *CG31690*, *CG32767*, *CG33981*, *CG4168*, *CG42322*, *CG42324*, *CG43901*, *CG5853*, *Diap1*, *dpr6*, *dpr8*, *E2f1*, *ed*, *Eip63E*, *FAM21*, *fipi*, *fred*, *fru*, *IA-2*, *Lac*, *Lmpt*, *lncRNA:CR32773*, *lncRNA:iab8*, *Moe*, *mtgo*, *nub*, *Pde9*, *PsGEF*, *Ptp99A*, *pum*, *Pvf3*, *rdgA*, *Rgk3*, *Src64B*) (MORGANTE *et al.* 2015), while a wing morphology screen shared 16 genes (*bru1*, *Bx*, *CG1358*, *CG14926*, *Con*, *dally*, *dar1*, *Dgk*, *ds*, *Dys*, *Egfr*, *Lar*, *luna*, *pip*, *RhoGEF64C*, *Sp1*) (PITCHERS *et al.* 2019). The overlap between these studies suggests that modifiers of robustness for flight performance also impact other traits, affirming their pleiotropic nature and their significance in quantitative genetic variation. These findings underscore

the importance of studying the genetic architecture of robustness as it can impact the outcome of natural selection on quantitative traits.

Conclusions

We present results from four analyses across four sex-based phenotypes surveying different facets of the genetic architecture of robustness for flight performance. Several of the variants were shared between sexes, though many more differed between them. Future studies in other phenotypes should consider evaluating both the mean and coefficient of variation for their focal phenotype to better understand modifiers affecting robustness in a specific complex trait, as well as robustness in complex traits more generally. Doing so would provide a better survey of the genetic modifiers of robustness as a phenotype and allow researchers to glean greater insight into the mechanisms of evolutionary change.

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Table 1. Different approaches uncover different types of genetic modifiers affecting the focal phenotype. No single screen will identify all modifiers, so four overlapping approaches were conducted to better survey the genetic architecture of robustness of flight performance.		
Screen type	Target modifier type	Analysis platform
Additive	Variants of large effect	DGRP2 webserver/FastLMM
Marginal	Interconnected variants	MAPIT
Epistatic interactions	Connections between variants	PLINK
Whole gene	Genes of moderate effect	PEGASUS_flies

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Table 2. Eight additive variants passed the Bonferroni threshold. In the additive approach, eight variants passed the strict Bonferroni significance threshold ($P \leq 2.63E-8$). These common variants were typically near the Minor Allele Frequency (MAF) threshold of 0.05. Nearly all variants mapped to genes, three of which had human homologs. Non-coding variants mapped to introns or upstream of the gene's coding region, however three variants also contained transcription factor binding sites (TFBS) annotated active during the embryonic stage (NEGRE *et al.* 2011).

Variant	MAF	Variant type	Annotation		
			Gene symbol		Embryonic TFBS
			Dmel	Hsap	
2L_7949902_SNP	0.053	Intron	<i>Snoo</i>	<i>SKI</i>	
2L_7949906_SNP	0.053	Intron	<i>Snoo</i>	<i>SKI</i>	-
2R_17433667_SNP	0.051	Intron	<i>Egfr</i>	<i>EGFR</i>	<i>bcd, da, dl, gt, hb, kni, Med, prd, sna, tll, twi, disco, Trl</i>
2R_17987191_SNP	0.067	Upstream (152 bp)	<i>Flapper/CG11073</i>	-	<i>dl</i>
2R_17987203_SNP	0.062	Upstream (164 bp)	<i>Flapper/CG11073</i>	-	<i>dl</i>
3L_8237797_DEL	0.084	Intron	<i>Dscam4</i>	<i>DSCAM</i>	-
3R_4379159_SNP	0.053	Non-synonymous	<i>Or85d</i>	-	-
3R_9684126_SNP	0.15	Intergenic		-	-

Table 3. Each analysis and sex-based phenotype identified varying enrichment for genetic modifiers. (A) Additive loci (variants and the genes they map to) at Bonferroni and traditional DGRP GWAS thresholds differ in enrichment by an order of magnitude. (B) Marginal variants mapped to several genes, and were tested for (C) epistatic interactions. Marginal variants were only tested for epistatic interactions if they passed $MAF \geq 0.05$ in the unimputed genome. Finally, (D) whole genes were identified consistently across all sex-based phenotypes				
(A) Additive Loci				
	Male	Female	Sex-Average	Sex-Different
Variants (Bonferroni; $P \leq 2.63e-8$)	2	5	4	0
Genes (Bonferroni; $P \leq 2.63e-8$)	1	3	3	0
Variants (Traditional DGRP; $P \leq 1.00e-5$)	75	76	76	21
Genes (Traditional DGRP; $P \leq 1.00e-5$)	49	62	58	17
(B) Marginal Loci				
	Male	Female	Sex-Average	Sex-Different
Variants (Bonferroni; $P \leq 2.56e-8$)	1	53	19	45
Genes (Bonferroni; $P \leq 2.56e-8$)	0	30	11	32
(C) Epistatic Loci				
	Male ($P \leq 2.63E-8$)	Female ($P \leq 5.06E-10$)	Sex-Average ($P \leq 1.34e-9$)	Sex-Different ($P \leq 5.90e-10$)
Paired Primary Variants	1	7	3	8
Paired Primary Genes	0	3	1	2
Paired Secondary Variants	20	428	611	2019
Paired Secondary Genes	13	193	243	763
(D) Gene-scores				
	Male	Female	Sex-Average	Sex-Different
Whole genes (Bonferroni; $P \leq 3.43E-6$)	10	22	20	10

<p>Table 4. Significant variants are non-uniformly distributed across site classes. Variants mapped to several site classes in the genome. Across all variant-based analyses, intergenic (variants lacking FBgn annotations) and genic (sites with FBgn annotations) sites were both represented. Some genic variants mapped to multiple FBgn (Genic—mapped). These genic variants mapped to 12 different site classes.</p>				
Site class	Additive ($P \leq 2.63E-8$)	Additive ($P \leq 1E-5$)	Marginal ($P \leq 2.56E-8$)	Epistatic (variable)
Intergenic	1	25	21	608
Genic—unique	7	138	66	2443
Genic—mapped	7	217	97	3275
Novel start site	0	1	0	9
Splice site region	0	0	0	1
Codon change plus codon insertion	0	0	0	2
Codon deletion	0	0	0	1
Non-synonymous coding	1	6	1	81
Exon (candidate region)	0	3	1	61
Synonymous coding	0	8	7	238
Upstream	2	47	9	462
Downstream	0	24	15	592
Intron	4	121	62	1683
5' UTR	0	1	0	50
3' UTR	0	6	2	95

Figure 1. The *Drosophila* Genetic Reference Panel lines demonstrate variation for robustness in flight performance across genotypes and sexes. (A) Flies were assayed for flight performance using a meter-long flight column (BABCOCK AND GANETZKY 2014). The coefficient of variation (mean-normalized standard deviation) is a proxy for robustness; more robust genotypes have less variation in landing height around the mean. Flies that passed through the column were excluded from the analysis. (B) The phenotypic distribution of sex-genotype pairs, ordered by increasing male score, demonstrates the DGRP lines have variation in their robustness for flight performance. Genotypes demonstrated phenotypic variation for robustness in both sexes. (C) Males were generally more robust than females, though the two were related ($R = 0.55$, $P < 1E-16$; regression line in red). Sexual dimorphism is observed by the intersection of the regression line and $y = x$ line (gray). (D) Additive variants in the sex-average analysis, visualized as a function of the $-\log_{10}$ of variants' P -value illustrates several variants (red) passed the traditional DGRP significance threshold ($P \leq 1E-5$; gray solid line), and three (red with black outline) passed Bonferroni significance threshold ($P \leq 2.63E-8$, gray dashed line). Variants that did not pass the significance threshold are colored in black or gray by chromosome.

Functional validation of candidate genes

Figure 2. Several genes validated for robustness of flight performance. Flies homozygous for *Mi{ET1}* insertion constructs inserted in candidate genes (experiment) were tested against their background control (control). Comparisons between control and experimental lines were assessed for significance using a Kolmogorov-Smirnoff test ($P \leq 0.05$; red points and bold text). Values to the left of the midline suggest control genotypes were more robust than experimental lines, while the opposite is true for values to the right of the line. (A) Seven constructs were significant in males, (B) while 13 were significant in females. Some candidate genes were tested more than once (*CadN*, *Dscam4*, and *flapper*) because they were strongly significant in the sex-average additive association screen. Separate constructs are denoted by a suffix containing a 'MB' code.

Figure 3. Several genetic variants positively associate with flight performance across different types of analyses. (A) Manhattan plot for sex-average whole gene analysis suggests several genes (red) were significant above a Bonferroni threshold ($P \leq 3.43E-6$, gray line). (B) Manhattan plot for sex-average marginal analysis suggests several variants (red) were significant above a Bonferroni threshold ($P \leq 2.56E-8$, gray line). For each plot, points are arranged by relative chromosome (genomic) position and all points are $-\log_{10}$ transformed.

Figure 4. Robustness of flight performance is comprised of an interconnected genetic architecture. (A) There were several interactions between genes identified from marginal variants. In particular, *PKC-δ* had the greatest number of interactions with other marginal genes, while *CG15651* and *CG9313* were next. There was a marginal variant that overlapped with *CG15651* and *CG9313*, so all edges connecting to *CG15651* also connect to *CG9313*, however there was an independent variant in *CG9313* that did not overlap with *CG15651* that interacted in the sex-difference screen with *PCK-δ* and *ush*. Intergenic regions that also interacted with genic marginal variants are not displayed. (B) For the additive, marginal, and epistatic variants identified, additive variants were unique, while marginal and epistatic variants had some overlap. This overlap was expected since the marginal variants served as a subset in searching for epistatic variants. (C) Genes and genes mapped from minSNP-variants had some overlap between analyses, though most genes were unique to a single analysis. When comparing variants and unique genes across (D-E) additive, marginal (F-G), and epistatic (H) analyses, there was roughly 15-20% overlap between the shared group and all those identified. However, there was no overlap between the (I) whole genes identified using *PEGASUS_flies*.

Literature Cited

- Abbott, J. K., S. Bedhomme and A. K. Chippindale, 2010 Sexual conflict in wing size and shape in *Drosophila melanogaster*. *Journal of Evolutionary Biology* 23: 1989-1997.
- Albayrak, C., C. A. Jordi, C. Zechner, J. Lin, C. A. Bichsel *et al.*, 2016 Digital Quantification of Proteins and mRNA in Single Mammalian Cells. *Molecular Cell* 61: 914-924.
- Ashburner, M., C. A. Ball, J. A. Blake, D. Botstein, H. Butler *et al.*, 2000 Gene Ontology: tool for the unification of biology. *Nature Genetics* 25: 25-29.
- Ayroles, J. F., S. M. Buchanan, C. O'Leary, K. Skutt-Kakaria, J. K. Grenier *et al.*, 2015 Behavioral idiosyncrasy reveals genetic control of phenotypic variability. *Proceedings of the National Academy of Sciences of the United States of America* 112: 6706-6711.
- Babcock, D. T., and B. Ganetzky, 2014 An Improved Method for Accurate and Rapid Measurement of Flight Performance in *Drosophila*. *Jove-Journal of Visualized Experiments*.
- Bellen, H. J., R. W. Levis, Y. C. He, J. W. Carlson, M. Evans-Holm *et al.*, 2011 The *Drosophila* Gene Disruption Project: Progress Using Transposons With Distinctive Site Specificities. *Genetics* 188: 731-U341.
- Benzer, S., 1973 GENETIC DISSECTION OF BEHAVIOR. *Scientific American* 229: 24-37.
- Berger, D., E. C. Berg, W. Widegren, G. Arnqvist and A. A. Maklakov, 2014 Multivariate intralocus sexual conflict in seed beetles. *Evolution* 68: 3457-3469.
- Berger, D., T. You, M. R. Minano, K. Grieshop, M. I. Lind *et al.*, 2016 Sexually antagonistic selection on genetic variation underlying both male and female same-sex sexual behavior. *Bmc Evolutionary Biology* 16.
- Brockington, M., Y. Yuva, P. Prandini, S. C. Brown, S. Torelli *et al.*, 2001 Mutations in the fukutin-related protein gene (FKRP) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. *Human Molecular Genetics* 10: 2851-2859.
- Browning, B. L., and S. R. Browning, 2016 Genotype Imputation with Millions of Reference Samples. *American Journal of Human Genetics* 98: 116-126.
- Browning, S. R., and B. L. Browning, 2007 Rapid and accurate haplotype phasing and missing-data inference for whole-genome association studies by use of localized haplotype clustering. *American Journal of Human Genetics* 81: 1084-1097.
- Carbon, S., E. Douglass, N. Dunn, B. Good, N. L. Harris *et al.*, 2019 The Gene Ontology Resource: 20 years and still GOing strong. *Nucleic Acids Research* 47: D330-D338.
- Chen, B., and A. Wagner, 2012 Hsp90 is important for fecundity, longevity, and buffering of cryptic deleterious variation in wild fly populations. *BMC evolutionary biology* 12: 25.
- Chintapalli, V. R., J. Wang and J. A. T. Dow, 2007 Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nature Genetics* 39: 715-720.

- Choi, Y., and A. P. Chan, 2015 PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics* 31: 2745-2747.
- Colomb, J., and B. Brembs, 2016 PKC in motoneurons underlies self learning, a form of motor learning in *Drosophila*. *Peerj* 4.
- Consortium, T. A. o. G. R., 2020 Alliance of Genome Resources Portal: unified model organism research platform. *Nucleic acids research* 48: D650-D658.
- Couto, A., M. Alenius and B. J. Dickson, 2005 Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Current Biology* 15: 1535-1547.
- Crawford, L., P. Zeng, S. Mukherjee and X. Zhou, 2017 Detecting epistasis with the marginal epistasis test in genetic mapping studies of quantitative traits. *Plos Genetics* 13.
- Cubadda, Y., P. Heitzler, R. P. Ray, M. Bourouis, P. Romain *et al.*, 1997 u-shaped encodes a zinc finger protein that regulates the proneural genes achaete and scute during the formation of bristles in *Drosophila*. *Genes & Development* 11: 3083-3095.
- Danecek, P., A. Auton, G. Abecasis, C. A. Albers, E. Banks *et al.*, 2011 The variant call format and VCFtools. *Bioinformatics* 27: 2156-2158.
- dos Santos, G., A. J. Schroeder, J. L. Goodman, V. B. Strelets, M. A. Crosby *et al.*, 2015 FlyBase: introduction of the *Drosophila melanogaster* Release 6 reference genome assembly and large-scale migration of genome annotations. *Nucleic Acids Research* 43: D690-D697.
- Edwards, A. C., L. Zwarts, A. Yamamoto, P. Callaerts and T. F. Mackay, 2009 Mutations in many genes affect aggressive behavior in *Drosophila melanogaster*. *BMC biology* 7: 29.
- Fedotov, S. A., J. V. Bragina, N. G. Besedina, L. V. Danilenkova, E. A. Kamysheva *et al.*, 2018 Gene CG15630 (*fipi*) is involved in regulation of the interpulse interval in *Drosophila* courtship song. *Journal of Neurogenetics* 32: 15-26.
- Frise, E., A. S. Hammonds and S. E. Celniker, 2010 Systematic image-driven analysis of the spatial *Drosophila* embryonic expression landscape. *Molecular Systems Biology* 6.
- Fromental-Romain, C., N. Taquet and P. Romain, 2010 Transcriptional interactions between the pannier isoforms and the cofactor U-shaped during neural development in *Drosophila*. *Mechanisms of Development* 127: 442-457.
- Furman, D., and T. Bukharina, 2008 How *Drosophila melanogaster* forms its mechanoreceptors. *Current genomics* 9: 312-323.
- Gaertner, B. E., E. A. Ruedi, L. J. McCoy, J. M. Moore, M. F. Wolfner *et al.*, 2015 Heritable Variation in Courtship Patterns in *Drosophila melanogaster*. *G3-Genes Genomes Genetics* 5: 531-539.
- Gaudet, P., M. S. Livstone, S. E. Lewis and P. D. Thomas, 2011 Phylogenetic-based propagation of functional annotations within the Gene Ontology consortium. *Briefings in Bioinformatics* 12: 449-462.
- Getahun, M. N., M. Thoma, S. Lavista-Llanos, I. Keeseey, R. A. Fandino *et al.*, 2016 Intracellular regulation of the insect chemoreceptor complex impacts odour localization in flying insects. *Journal of Experimental Biology* 219: 3428-3438.

- Ghosh, A., E. J. Rideout and S. S. Grewal, 2014 TIF-IA-Dependent Regulation of Ribosome Synthesis in *Drosophila* Muscle Is Required to Maintain Systemic Insulin Signaling and Larval Growth. *Plos Genetics* 10.
- Gorostiza, E. A., J. Colomb and B. Brembs, 2016 A decision underlies phototaxis in an insect. *Open Biology* 6.
- Grumblin, G., V. Strelets and C. FlyBase, 2006 FlyBase: anatomical data, images and queries. *Nucleic Acids Research* 34: D484-D488.
- Hesselberg, T., and F. O. Lehmann, 2009 The role of experience in flight behaviour of *Drosophila*. *Journal of Experimental Biology* 212: 3377-3386.
- Hiesinger, P. R., and B. A. Hassan, 2018 The Evolution of Variability and Robustness in Neural Development. *Trends in Neurosciences* 41: 577-586.
- Honegger, K., and B. de Bivort, 2018 Stochasticity, individuality and behavior. *Current Biology* 28: R8-R12.
- Hong, W. Z., T. J. Mosca and L. Q. Luo, 2012 Teneurins instruct synaptic partner matching in an olfactory map. *Nature* 484: 201-+.
- Hu, Y. H., I. Flockhart, A. Vinayagam, C. Bergwitz, B. Berger *et al.*, 2011 An integrative approach to ortholog prediction for disease-focused and other functional studies. *Bmc Bioinformatics* 12.
- Huang, W., A. Massouras, Y. Inoue, J. Peiffer, M. Ramia *et al.*, 2014 Natural variation in genome architecture among 205 *Drosophila melanogaster* Genetic Reference Panel lines. *Genome Research* 24: 1193-1208.
- Huang, W., S. Richards, M. A. Carbone, D. H. Zhu, R. R. H. Anholt *et al.*, 2012 Epistasis dominates the genetic architecture of *Drosophila* quantitative traits. *Proceedings of the National Academy of Sciences of the United States of America* 109: 15553-15559.
- Jovaisaite, V., L. Mouchiroud and J. Auwerx, 2014 The mitochondrial unfolded protein response, a conserved stress response pathway with implications in health and disease. *Journal of Experimental Biology* 217: 137-143.
- Kain, J. S., C. Stokes and B. L. de Bivort, 2012 Phototactic personality in fruit flies and its suppression by serotonin and white. *Proceedings of the National Academy of Sciences of the United States of America* 109: 19834-19839.
- Kise, Y., and D. Schmucker, 2013 Role of self-avoidance in neuronal wiring. *Current Opinion in Neurobiology* 23: 983-989.
- Klingenberg, C. P., 2019 Phenotypic Plasticity, Developmental Instability, and Robustness: The Concepts and How They Are Connected. *Frontiers in Ecology and Evolution* 7.
- Lajus, D. L., P. V. Golovin, A. O. Yurtseva, T. S. Ivanova, A. S. Dorgham *et al.*, 2019 Fluctuating asymmetry as an indicator of stress and fitness in stickleback: a review of the literature and examination of cranial structures. *Evolutionary Ecology Research* 20.
- Lehmann, F. O., and J. Bartussek, 2017 Neural control and precision of flight muscle activation in *Drosophila*. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology* 203: 1-14.
- Li, J. F., S. Han, H. J. Li, N. D. Udeshi, T. Svinkina *et al.*, 2020 Cell-Surface Proteomic Profiling in the Fly Brain Uncovers Wiring Regulators. *Cell* 180: 373-+.

- Li, X. Y., S. MacArthur, R. Bourgon, D. Nix, D. A. Pollard *et al.*, 2008 Transcription factors bind thousands of active and inactive regions in the *Drosophila* blastoderm. *Plos Biology* 6: 365-388.
- Long, T. A. F., and W. R. Rice, 2007 Adult locomotory activity mediates intralocus sexual conflict in a laboratory-adapted population of *Drosophila melanogaster*. *Proceedings of the Royal Society B-Biological Sciences* 274: 3105-3112.
- MacArthur, S., X. Y. Li, J. Y. Li, J. B. Brown, H. C. Chu *et al.*, 2009 Developmental roles of 21 *Drosophila* transcription factors are determined by quantitative differences in binding to an overlapping set of thousands of genomic regions. *Genome Biology* 10.
- Mackay, T. F., and W. Huang, 2018 Charting the genotype–phenotype map: lessons from the *Drosophila melanogaster* Genetic Reference Panel. *Wiley Interdisciplinary Reviews: Developmental Biology* 7.
- Mackay, T. F. C., S. Richards, E. A. Stone, A. Barbadilla, J. F. Ayroles *et al.*, 2012 The *Drosophila melanogaster* Genetic Reference Panel. *Nature* 482: 173-178.
- Marcus, J. M., 2001 The development and evolution of crossveins in insect wings. *Journal of Anatomy* 199: 211-216.
- Menezes, B. F., J. Salces-Ortiz, H. Muller, N. Burlet, S. Martinez *et al.*, 2018 An attempt to select non-genetic variation in resistance to starvation and reduced chill coma recovery time in *Drosophila melanogaster*. *Journal of Experimental Biology* 221.
- Metaxakis, A., S. Oehler, A. Klinakis and C. Savakis, 2005 Minos as a genetic and genomic tool in *Drosophila melanogaster*. *Genetics* 171: 571-581.
- Milan, M., F. J. Diaz-Benjumea and S. M. Cohen, 1998 Beadex encodes an LMO protein that regulates Apterous LIM-homeodomain activity in *Drosophila* wing development: a model for LMO oncogene function. *Genes & Development* 12: 2912-2920.
- Morgante, F., P. Sorensen, D. A. Sorensen, C. Maltecca and T. F. C. Mackay, 2015 Genetic Architecture of Micro-Environmental Plasticity in *Drosophila melanogaster*. *Scientific Reports* 5.
- Moses, A. M., D. A. Pollard, D. A. Nix, V. N. Iyer, X. Y. Li *et al.*, 2006 Large-scale turnover of functional transcription factor binding sites in *Drosophila*. *Plos Computational Biology* 2: 1219-1231.
- Mossman, J. A., L. M. Biancani, C. T. Zhu and D. M. Rand, 2016 Mitonuclear Epistasis for Development Time and Its Modification by Diet in *Drosophila*. *Genetics* 203: 463-+.
- Muhammad, K., S. Reddy-Alla, J. H. Driller, D. Schreiner, U. Rey *et al.*, 2015 Presynaptic spinophilin tunes neurexin signalling to control active zone architecture and function. *Nature Communications* 6.
- Munoz-Munoz, F., V. P. Carreira, N. Martinez-Abadias, V. Ortiz, R. Gonzalez-Jose *et al.*, 2016 *Drosophila* wing modularity revisited through a quantitative genetic approach. *Evolution* 70: 1530-1541.
- Nagai, T., and K. Mizuno, 2014 Multifaceted roles of Furry proteins in invertebrates and vertebrates. *Journal of Biochemistry* 155: 137-146.
- Nakka, P., B. J. Raphael and S. Ramachandran, 2016 Gene and Network Analysis of Common Variants Reveals Novel Associations in Multiple Complex Diseases. *Genetics* 204: 783-+.

- Negre, N., C. D. Brown, L. J. Ma, C. A. Bristow, S. W. Miller *et al.*, 2011 A cis-regulatory map of the *Drosophila* genome. *Nature* 471: 527-531.
- Paul, L., S. H. Wang, S. N. Manivannan, L. Bonanno, S. Lewis *et al.*, 2013 Dpp-induced Egfr signaling triggers postembryonic wing development in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America* 110: 5058-5063.
- Pavlou, H. J., and S. F. Goodwin, 2013 Courtship behavior in *Drosophila melanogaster*: towards a 'courtship connectome'. *Current Opinion in Neurobiology* 23: 76-83.
- Pellegrino, M. W., A. M. Nargund and C. M. Haynes, 2013 Signaling the mitochondrial unfolded protein response. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1833: 410-416.
- Pitchers, W., J. Nye, E. J. Marquez, A. Kowalski, I. Dworkin *et al.*, 2019 A Multivariate Genome-Wide Association Study of Wing Shape in *Drosophila melanogaster*. *Genetics* 211: 1429-1447.
- Purcell, S., B. Neale, K. Todd-Brown, L. Thomas, M. A. R. Ferreira *et al.*, 2007 PLINK: A tool set for whole-genome association and population-based linkage analyses. *American Journal of Human Genetics* 81: 559-575.
- Quijano, J. C., M. J. Stinchfield, B. Zerlanko, Y. Y. Gibbens, N. T. Takaesu *et al.*, 2010 The Sno Oncogene Antagonizes Wingless Signaling during Wing Development in *Drosophila*. *Plos One* 5.
- Quinto-Sanchez, M., F. Munoz-Munoz, J. Gomez-Valdes, C. Cintas, P. Navarro *et al.*, 2018 Developmental pathways inferred from modularity, morphological integration and fluctuating asymmetry patterns in the human face. *Scientific Reports* 8.
- Roman, A. C., J. Vicente-Page, A. Perez-Escudero, J. M. Carvajal-Gonzalez, P. M. Fernandez-Salguero *et al.*, 2018 Histone H4 acetylation regulates behavioral inter-individual variability in zebrafish. *Genome Biology* 19.
- Romero-Calderon, R., R. M. Shome, A. F. Simon, R. W. Daniels, A. DiAntonio *et al.*, 2007 A screen for neurotransmitter transporters expressed in the visual system of *Drosophila melanogaster* identifies three novel genes. *Developmental Neurobiology* 67: 550-569.
- Rutherford, S. L., and S. Lindquist, 1998 Hsp90 as a capacitor for morphological evolution. *Nature* 396: 336-342.
- Sadaf, S., O. V. Reddy, S. P. Sane and G. Hasan, 2015 Neural Control of Wing Coordination in Flies. *Current Biology* 25: 80-86.
- Sambandan, D., A. Yamamoto, J. J. Fanara, T. F. C. Mackay and R. R. H. Anholt, 2006 Dynamic genetic interactions determine odor-guided behavior in *Drosophila melanogaster*. *Genetics* 174: 1349-1363.
- Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair *et al.*, 2012 Fiji: an open-source platform for biological-image analysis. *Nature Methods* 9: 676-682.
- Schnorrer, F., C. Schonbauer, C. C. H. Langer, G. Dietzl, M. Novatchkova *et al.*, 2010 Systematic genetic analysis of muscle morphogenesis and function in *Drosophila*. *Nature* 464: 287-291.
- Schor, I. E., J. F. Degner, D. Harnett, E. Cannavo, F. P. Casale *et al.*, 2017 Promoter shape varies across populations and affects promoter evolution and expression noise. *Nature Genetics* 49: 550-+.

- Shirangi, T. R., A. M. Wong, J. W. Truman and D. L. Stern, 2016 Doublesex Regulates the Connectivity of a Neural Circuit Controlling *Drosophila* Male Courtship Song. *Developmental Cell* 37: 533-544.
- Siepel, A., G. Bejerano, J. S. Pedersen, A. S. Hinrichs, M. M. Hou *et al.*, 2005 Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. *Genome Research* 15: 1034-1050.
- Siepel, A., and D. Haussler, 2005 Phylogenetic hidden Markov models, pp. 325-351 in *Statistical methods in molecular evolution*. Springer.
- Skeath, J. B., and S. B. Carroll, 1991 REGULATION OF ACHAETE-SCUTE GENE-EXPRESSION AND SENSORY ORGAN PATTERN-FORMATION IN THE *DROSOPHILA* WING. *Genes & Development* 5: 984-995.
- Soto, I. M., V. P. Carreira, E. M. Soto and E. Hasson, 2008 Wing morphology and fluctuating asymmetry depend on the host plant in cactophilic *Drosophila*. *Journal of Evolutionary Biology* 21: 598-609.
- Spierer, A. N., J. A. Mossman, S. P. Smith, L. Crawford, S. Ramachandran *et al.*, 2020 Natural variation in the regulation of neurodevelopmental genes modifies flight performance in *Drosophila*. *bioRxiv*: 2020.2005.2027.118604.
- Sztepanacz, J. L., K. McGuigan and M. W. Blows, 2017 Heritable Micro-environmental Variance Covaries with Fitness in an Outbred Population of *Drosophila serrata*. *Genetics* 206: 2185-2198.
- Tadros, W., S. W. Xu, O. Akin, C. H. Yi, G. J. E. Shin *et al.*, 2016 Dscam Proteins Direct Dendritic Targeting through Adhesion. *Neuron* 89: 480-493.
- Thistle, R., P. Cameron, A. Ghorayshi, L. Dennison and K. Scott, 2012 Contact chemoreceptors mediate male-male repulsion and male-female attraction during *Drosophila* courtship. *Cell* 149: 1140-1151.
- Thomas, S., X. Y. Li, P. J. Sabo, R. Sandstrom, R. E. Thurman *et al.*, 2011 Dynamic reprogramming of chromatin accessibility during *Drosophila* embryo development. *Genome Biology* 12.
- Thurmond, J., J. L. Goodman, V. B. Strelets, H. Attrill, L. S. Gramates *et al.*, 2019 FlyBase 2.0: the next generation. *Nucleic Acids Research* 47: D759-D765.
- Tilney, L. G., P. S. Connelly, L. Ruggiero, K. A. Vranich and G. M. Guild, 2003 Actin filament turnover regulated by cross-linking accounts for the size, shape, location, and number of actin bundles in *Drosophila* bristles. *Molecular Biology of the Cell* 14: 3953-3966.
- Tootle, T. L., D. Williams, A. Hubb, R. Frederick and A. Spradling, 2011 *Drosophila* Eggshell Production: Identification of New Genes and Coordination by Pxt. *Plos One* 6.
- Tsuzuki, S., M. Ochiai, H. Matsumoto, S. Kurata, A. Ohnishi *et al.*, 2012 *Drosophila* growth-blocking peptide-like factor mediates acute immune reactions during infectious and non-infectious stress. *Scientific Reports* 2.
- Turner, T. L., P. M. Miller and V. A. Cochrane, 2013 Combining Genome-Wide Methods to Investigate the Genetic Complexity of Courtship Song Variation in *Drosophila melanogaster*. *Molecular Biology and Evolution* 30: 2113-2120.
- Valen, L. V., 1962 A study of fluctuating asymmetry. *Evolution* 16: 125-142.

- 822 Vogt, G., 2015 Stochastic developmental variation, an epigenetic source of phenotypic
823 diversity with far-reaching biological consequences. *Journal of Biosciences* 40:
824 159-204.
- 825 Vogt, G., M. Huber, M. Thiemann, G. van den Boogaart, O. J. Schmitz *et al.*, 2008
826 Production of different phenotypes from the same genotype in the same
827 environment by developmental variation. *Journal of Experimental Biology* 211:
828 510-523.
- 829 Wagner, A., 2008 Robustness and evolvability: a paradox resolved. *Proceedings of the*
830 *Royal Society B-Biological Sciences* 275: 91-100.
- 831 Wasbrough, E. R., S. Dorus, S. Hester, J. Howard-Murkin, K. Lilley *et al.*, 2010 The
832 *Drosophila melanogaster* sperm proteome-II (DmSP-II). *Journal of proteomics*
833 73: 2171-2185.
- 834 Wrischnik, L. A., J. R. Timmer, L. A. Megna and T. W. Cline, 2003 Recruitment of the
835 proneural gene *scute* to the *Drosophila* sex-determination pathway. *Genetics*
836 165: 2007-2027.
- 837 Yatsu, J., M. Hayashi, M. Mukai, K. Arita, S. Shigenobu *et al.*, 2008 Maternal RNAs
838 encoding transcription factors for germline-specific gene expression in
839 *Drosophila* embryos. *International Journal of Developmental Biology* 52: 913-
840 923.
- 841 Yu, J. Y., M. I. Kanai, E. Demir, G. Jefferis and B. J. Dickson, 2010 Cellular
842 Organization of the Neural Circuit that Drives *Drosophila* Courtship Behavior.
843 *Current Biology* 20: 1602-1614.
- 844 zur Lage, P., F. G. Newton and A. P. Jarman, 2019 Survey of the Ciliary Motility
845 Machinery of *Drosophila* Sperm and Ciliated Mechanosensory Neurons Reveals
846 Unexpected Cell-Type Specific Variations: A Model for Motile Ciliopathies.
847 *Frontiers in Genetics* 10.