Gene Protein Sequence Evolution Can Predict the Rapid Divergence of Ovariole Numbers in the *Drosophila melanogaster* Subgroup

Carrie A. Whittle [1,2] and Cassandra G. Extavour [1,2,3,*]

[1]Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA
[2]Howard Hughes Medical Institute, Chevy Chase, MD, USA
[3]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA

*Corresponding author: E-mail: extavour@oeb.harvard.edu.

Accepted: May 30, 2024

Abstract

Ovaries play key roles in fitness and evolution: they are essential female reproductive structures that develop and house the eggs in sexually reproducing animals. In *Drosophila*, the mature ovary contains multiple tubular egg-producing structures known as ovarioles. Ovarioles arise from somatic cellular structures in the larval ovary called terminal filaments (TFs), formed by TF cells and subsequently enclosed by sheath (SH) cells. As in many other insects, ovariole number per female varies extensively in *Drosophila*. At present, however, there is a striking gap of information on genetic mechanisms and evolutionary forces that shape the well-documented rapid interspecies divergence of ovariole numbers. To address this gap, here we studied genes associated with *Drosophila melanogaster* ovariole number or functions based on recent experimental and transcriptional datasets from larval ovaries, including TFs and SH cells, and assessed their rates and patterns of molecular evolution in five closely related species of the *melanogaster* subgroup that exhibit species-specific differences in ovariole numbers. From comprehensive analyses of protein sequence evolution (dN/dS), branch-site positive selection, expression specificity (*tau*), and phylogenetic regressions (phylogenetic generalized least squares), we report evidence of 42 genes that showed signs of playing roles in the genetic basis of interspecies evolutionary change of *Drosophila* ovariole number. These included the signaling genes *upd2* and *Ilp5* and extracellular matrix genes *vkg* and *Col4a1*, whose dN/dS predicted ovariole numbers among species. Together, we propose a model whereby a set of ovariole-involved gene proteins have an enhanced evolvability, including adaptive evolution, facilitating rapid shifts in ovariole number among *Drosophila* species.

Key words: ovariole number, *Drosophila*, genetic mechanism, phenotype, dN/dS, adaptive evolution, *tau*.

Significance

Ovaries in *Drosophila*, like in other insects, contain egg-producing structures, known as ovarioles. The number of ovarioles per female varies among *Drosophila* species, but little is known about the genes and evolutionary dynamics that may shape interspecies changes in ovariole numbers. Here, we used a priori experimental and transcriptome data from *Drosophila melanogaster* to identify genes involved in ovariole formation and functions and studied their molecular evolution among its closely related species within the *melanogaster* subgroup. Using a multilayered analysis consisting of protein sequence divergence (dN/dS), adaptive evolution, expression breadth, and phylogenetic regressions, we identified 42 genes whose molecular evolution patterns were well linked to ovariole number divergence. Further, gene protein sequence divergence was often predictive of species ovariole numbers.

Introduction

Ovarian development is a process that is poised to play key roles in organismal evolutionary biology, as the female gonads form and house the oocytes and/or eggs that are central to fertility and reproductive success of a species, and thus affect their fitness (Miller et al. 2014; Macagno et al. 2015). In insects, the most well-studied model with respect to ovarian development and genetics is the fruit fly *Drosophila melanogaster* (Dansereau and Lasko 2008; Eliazer and Buszczak 2011; Li et al. 2014; Slaidina, Gupta et al. 2020; Lebo and McCall 2021). The mature ovary in *D. melanogaster*, as in other species of insects, is comprised of tubular egg-producing structures known as ovarioles (King et al. 1968; Dansereau and Lasko 2008; Lebo and McCall 2021), which are a central factor shaping organismal reproductive output (Montague et al. 1981; Starmer et al. 2003; Church et al. 2021). The number of ovarioles contained in the ovaries is highly variable within the genus *Drosophila* (Kambysellis and Heed 1971; Hodin and Riddiford 2000; Starmer et al. 2003; Markow et al. 2009; Sarikaya et al. 2019; Church et al. 2021). As an example, within the *melanogaster* subgroup, *D. melanogaster* has typically about 19 ovarioles per ovary, while its closely related sister species *Drosophila sechellia* has only about 8 to 9 ovarioles per ovary (Hodin and Riddiford 2000). A broad range of ovariole numbers has been observed across the family *Drosophilidae*, from one to more than 50 per ovary across the genus *Drosophila* (Sarikaya et al. 2019; Church et al. 2021). At present, however, we know little about the genetic basis of the evolution of ovariole number within insects (Hodin and Riddiford 2000; Markow et al. 2009; Sarikaya et al. 2019).

A central factor that may underlie the rapid interspecies transitions in ovariole numbers in *Drosophila* is the evolvability of ovariole-related protein-coding genes, that is, the tendency of the proteins encoded by these genes to diverge and/or undergo adaptive sequence changes (Wagner and Zhang 2011; Cutter and Bundus 2020). Functional amino acid changes in protein-coding DNA and associated selection pressures (measured as nonsynonymous to synonymous changes, or dN/ds; Bielawski and Yang 2005; Cutter and Bundus 2020; Yang 1997) can play a significant role in shaping interspecies divergence of developmental processes and other key phenotypes (Hoekstra and Coyne 2007). For instance, dN/dS of specific genes or sets of genes has been correlated with the divergence of sperm length in *Drosophila* (Chebbo et al. 2021), sperm head size (Luke et al. 2014) and testis size (Ramm et al. 2008) in rodents, plumage color in toucans (Corso et al. 2016), and brain mass in primates (Montgomery et al. 2011), as well as other species traits (Swanson and Vacquier 2002; Hoekstra and Coyne 2007; Clark et al. 2009; Cutter and Bundus 2020). Several lines of evidence indicate that ovariole number may also be a phenotype whose interspecies evolution in *Drosophila* is shaped by gene protein sequence changes and associated selection pressures (dN/dS; Yang and Nielsen 2002; Bielawski and Yang 2005; Yang 2007). Specifically, ovariole number is highly heritable and polygenic (Coyne et al. 1991; Wayne and McIntyre 2002; Bergland et al. 2008; Green and Extavour 2012; Sarikaya and Extavour 2015; Lobell et al. 2017; Kumar et al. 2020), and thus, genetic mechanisms exist wherein changes in ovariole-related gene protein products could lead to interspecies differences in ovariole numbers. Further, in *Drosophila*, sexual (positive) selection pressures have been commonly observed and mating behaviors are variable among taxa (Kaneshiro and Boake 1987; Singh et al. 2002; Singh and Singh 2014; Lupold et al. 2016; Wigby et al. 2020). These factors have been linked to accelerated interspecies protein sequence evolution in reproduction-related gene proteins and reproductive characteristics (Markow 2002; Swanson et al. 2004; Jagadeeshan and Singh 2005; Haerty et al. 2007; Kang et al. 2016), which may potentially include ovariole numbers. Natural adaptive selection may also influence ovariole number evolution in *Drosophila*. For example, ovariole numbers and/or functions among species have been correlated with local environmental conditions and with oviposition and larval substrates in the *melanogaster* subgroup as well as in the Hawaiian *Drosophila* (Kambysellis and Heed 1971; Kambysellis et al. 1995; Sarikaya et al. 2019). Finally, species-specific ovariole number may also be partly influenced by neutral protein sequence changes via random genetic drift (Kimura 1989; Kambysellis et al. 1995). For these reasons, we sought to investigate whether evolutionary pressures on changes in proteins (dN/dS) involved in ovariole formation and function, especially in those genes that exhibit signs of evolvability and adaptive evolution, could underlie or even predict interspecies divergence in ovariole number, as is the case for certain other fitness-related phenotypes in animals (Montgomery et al. 2011; Wagner and Zhang 2011; Luke et al. 2014; Corso et al. 2016; Chebbo et al. 2021).

The most crucial developmental period that determines ovariole number in *D. melanogaster* is the larval stage (Fig. 1) (King et al. 1968; Godt and Laski 1995; Hodin and Riddiford 2000; Sarikaya et al. 2012; Sarikaya and Extavour 2015; Slaidina, Gupta et al. 2020). Somatic gonad precursors specified during embryogenesis give rise to many different somatic ovarian cell types in the larval stage, and the numbers and behaviors of these somatic cells largely determine final ovariole number (Extavour and Akam 2003; Clark et al. 2007; Dansereau and Lasko 2008). Specifically, the number of terminal filaments (TFs; Fig. 1a), which are stacks of flattened intercalated TF cells in the anterior ovary at the late third larval instar stage (LL3), determines adult ovariole number (King et al. 1968; Godt and Laski 1995; Dansereau and Lasko 2008; Sarikaya et al. 2012; Sarikaya and Extavour 2015). Each TF is the starting point for formation of a single ovariole (Fig. 1b; Sahut-Barnola et al. 1996; Sarikaya et al. 2012), which contains an anterior germarium housing germ line stem cells

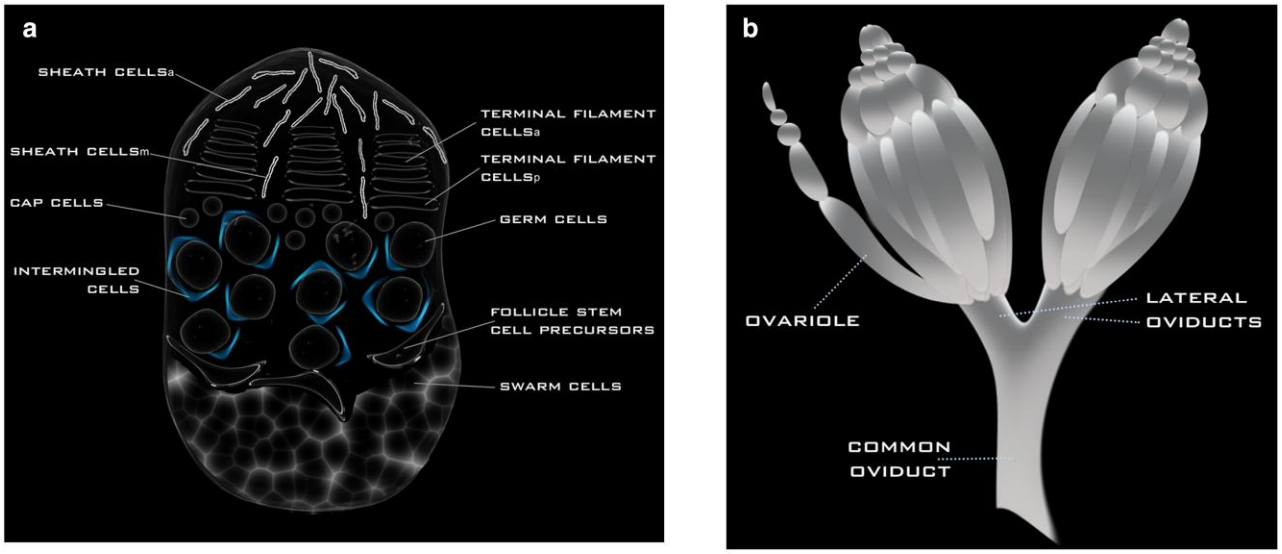

Fig. 1.—A schematic diagram of a) the late third-instar larval ovary with its germ cells and various somatic cell types and b) an external view of an adult ovary showing the ovarioles in each of the two ovaries that converge to the common oviduct in *D. melanogaster*. The relative cell positioning of cells in (a) is as denoted by Slaidina et al. (2020). For orientation, anterior is up in both panels.

and egg chambers that form the oocytes in an anterior-to-posterior pattern of oocyte maturation (Sahut-Barnola et al. 1996; Eliazer and Buszczak 2011; Sarikaya et al. 2012; Lebo and McCall 2021; Slaidina et al. 2021). Single-celled RNA sequencing (sc-RNA-seq) data (Slaidina, Banisch et al. 2020) suggest that LL3 TFs have anterior (TFa) and posterior (TFp) subgroups with distinct transcriptional profiles (Fig. 1a). Another key somatic cell type are the sheath (SH) cells, also located at the anterior of the LL3 ovary (Fig. 1a), and are subcategorized based on sc-RNA-seq into anterior sheath cells (SHa) and migrating sheath cells (SHm) (Slaidina, Gupta et al. 2020). The latter cells migrate in an anterior-to-posterior direction between the TFs, depositing basement membrane that partitions the remaining cells of the ovary (germ cells [GC] and posterior somatic cells) into the developing ovarioles (King et al. 1968; King 1970; Slaidina, Gupta et al. 2020). Additional somatic cells in the LL3 ovary include intermingled cells (IC), which are interspersed between the GC and are involved in their proliferation (Gilboa and Lehmann 2006), cap cells (CC), which form the adult germ line stem cell niche (Song et al. 2002), follicle stem cell precursors (FSCP), which give rise to adult follicle stem cells (Slaidina, Gupta et al. 2020; Slaidina et al. 2021), and swarm (SW) cells, whose precise functions largely remain to be ascertained (Slaidina, Gupta et al. 2020) (Fig. 1a). In this regard, understanding the interspecies evolution of ovariole number in *Drosophila* requires consideration of the genes and proteins regulating cell behavior in the larval ovary, and particularly the behaviors of the TF and SH cells, which are instrumental to determining ovariole numbers in *D. melanogaster*.

Until recently, research on the relationships between divergence in gene sequences and ovariole numbers in *Drosophila* was challenged by the lack of data on the identity of protein-coding genes expressed in somatic cells of the larval ovary that regulate ovariole number (Sarikaya et al. 2012; Sarikaya and Extavour 2015). Recently available large-scale functional genetic and cell type-specific expression data from *D. melanogaster*, however, now provide a means to systematically identify genes linked to ovariole numbers, and, in turn, assess their molecular evolution across species. A large-scale RNAi screen of 463 signaling genes from 14 conserved animal signaling pathways revealed that TF-mediated ovariole number determination is regulated by all conserved animal signaling pathways, and identified a specific set of genes affecting ovariole numbers and functions (Kumar et al. 2020). Another study using bulk RNA-seq expression data from GC and somatic cells separated by fluorescence-activated cell sorting (FACS) revealed additional genes differentially expressed throughout TF formation, suggesting their potential involvement in ovariole number regulation (Tarikere et al. 2022). In addition to those studies, a recent sc-RNA-seq study yielded unique transcriptional profiles for all of the known cell types in the *D. melanogaster* LL3 ovaries (Fig. 1), providing a novel resource to identify and study the evolution of genes transcribed in TF and SH cells, the two crucial cell types in determining ovariole number (Slaidina, Gupta et al. 2020).

Collectively, these datasets provide valuable empirical data from which to a priori identify sets of genes that

regulate ovariole numbers or functions in *Drosophila*, and, in turn, to evaluate which of these genes exhibit elevated or otherwise unusual rates of interspecies protein sequence evolution, including adaptive evolution, suggesting them as candidates for driving interspecies divergence of ovariole numbers in *Drosophila*. For example, by assessing dN/dS, we may ask whether ovariole-related gene protein sequences typically have been under strict purifying selection, which could mean that phenotypes regulated by these genes are likely to show high pleiotropy and low evolvability and to have minimal potential to diverge neutrally or adaptively (Fisher 1930; Otto 2004; Wagner and Zhang 2011; Cutter and Bundus 2020; Munds et al. 2021). If, in contrast, some ovariole-related genes have been subjected to relaxed selection and/or have commonly experienced adaptive changes, we might expect high phenotypic evolvability and adaptability (Otto 2004; Larracuente et al. 2008; Clark et al. 2009; Mank and Ellegren 2009; Montgomery et al. 2011; Luke et al. 2014; Corso et al. 2016; Chebbo et al. 2021). In this regard, the study of the evolution of protein-coding genes (from dN/dS) that are prescreened for likely roles in ovariole numbers and/or functions by studies like the ones described above (Kumar et al. 2020; Slaidina, Gupta et al. 2020; Tarikere et al. 2022) provides a novel pathway to advance our understanding of the genetic factors and evolutionary forces that shape rapid interspecies divergence in ovariole numbers.

In the present study, we rigorously assess the molecular evolutionary patterns of genes that regulate ovariole numbers and/or functions that were identified a priori based on one or both of functional genetic evidence (Kumar et al. 2020) or transcriptional activity (Slaidina, Gupta et al. 2020; Tarikere et al. 2022). We focus on the molecular evolution of ovariole-related genes within five species of the *melanogaster* subgroup of *Drosophila*, which is a closely related species clade that includes *D. melanogaster*, diverged from a common ancestor about 13 Mya (Tamura et al. 2004), and exhibits substantial interspecies variation in ovariole numbers (Hodin and Riddiford 2000; Starmer et al. 2003; Markow et al. 2009). From our assessments, we identify 42 genes that are high-confidence candidates for contributing to the genetic basis of interspecies divergence in ovariole numbers. We hypothesize that evolved changes in these genes are apt to underlie ovariole number divergence among taxa given that they exhibit an ovariole-related function (Kumar et al. 2020; Slaidina, Gupta et al. 2020; Tarikere et al. 2022), have a propensity to diverge in protein sequence, or high evolvability, show a high frequency of adaptive sequence evolution events in branches of the phylogeny, and are often associated with low pleiotropy (Yanai et al. 2005). Further, phylogenetic regressions show gene dN/dS has predictive associations to ovariole numbers. Collectively, our findings provide a genetic framework to explain the rapid interspecies divergence of ovariole numbers in *Drosophila*, which we propose is largely mediated by selection pressures shaping the evolution of functional protein sequences, and thus ovariole numbers.

Results

The Clade Under Study, the *melanogaster* Subgroup

For our study, we focused on a multilayered analysis of the molecular evolution of ovariole-related genes across five species from the *melanogaster* subgroup of *Drosophila*: *Drosophila simulans* (Dsim), *D. sechellia* (Dsec), *D. melanogaster* (Dmel), *Drosophila yakuba* (Dyak), and *Drosophila erecta* (Dere) (Fig. 2; *Drosophila ananassae* of the *melanogaster* group was used as an outgroup for phylogeny construction, see "*Drosophila* Phylogeny" section; the abbreviated names are used in tables and figures). Using this closely related species clade, we hypothesize that if genes with demonstrated roles in regulating ovariole numbers or formation are involved in the interspecies divergence of ovariole numbers, then they will exhibit relatively rapid evolution (dN/dS) as compared to the genome, as well as interspecies variation in dN/dS, signs of positive selection, and low pleiotropy (as inferred by high *tau* across tissues, supplementary table S1,

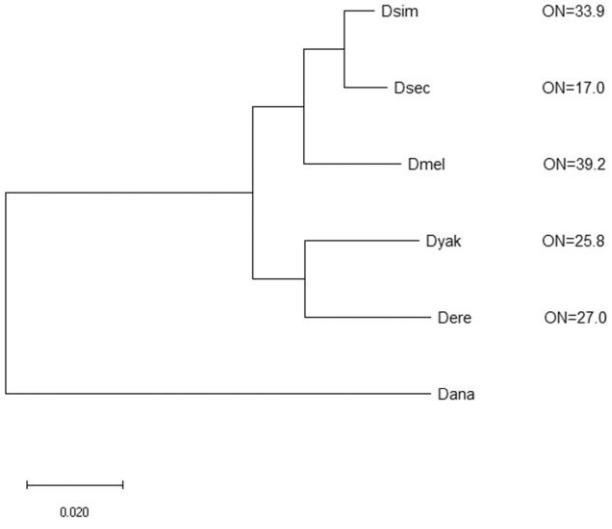

Fig. 2.—The phylogeny showing the five-species *melanogaster* subgroup under study that was based on a ML tree generated in MEGA v. 11 (Tamura, et al. 2021) and DNA sequence data from DrosoPhyla (Finet, et al. 2021). The five species of the *melanogaster* subgroup are shown. The relatively distantly related *D. ananassae* (Dana) was used as an outgroup for tree construction. Ovariole numbers are shown and are for two ovaries per female and from the following sources: *D. melanogaster* (Dmel), *D. sechellia* (Dsec), and *D. yakuba* (Dyak) (Hodin and Riddiford 2000), *D. simulans* (Dsim) (averaged, (Hodin and Riddiford 2000; Starmer, et al. 2003), and *D. erecta* (Dere) (Markow, et al. 2009) (see respective articles for variation). All nodes had 100/100 bootstrap support. ON, ovariole numbers.

Supplementary Material online). We further hypothesize that if evolutionary variation in these genes contributes to the genetic basis of evolved shifts in ovariole number, that dN/dS values for these genes may predict species ovariole numbers.

The five-species clade of *melanogaster* subgroup had the following advantages for our study: (i) all species within the clade are very closely related to *D. melanogaster* (Tamura et al. 2004; Obbard et al. 2012), the species for which experimental and transcriptome data on genes associated with ovariole numbers or functions are available (Kumar et al. 2020; Slaidina, Gupta et al. 2020; Tarikere et al. 2022), and thus, we hypothesize they are likely to share similarities in the genetic pathways affecting ovariole numbers, more so than we would be expected for distantly related species; (ii) the clade exhibits substantial variation in ovariole numbers among species, typically about 39.2 (per female) for *D. melanogaster* and 17.0 for *D. sechellia* and intermediate values for *D. simulans* (33.9), *D. yakuba* (25.8), and *D. erecta* (27.0) (Fig. 2; see values and variability; Hodin and Riddiford 2000; Starmer et al. 2003; Markow et al. 2009), and includes some species with similar ovariole numbers and others that markedly differ; (iii) the phylogeny is highly resolved (Fig. 2 (Cutter 2008; Obbard et al. 2012), unlike some other *Drosophila* clades and branches (Finet et al. 2021), and all five species are very closely related to each other (Tamura et al. 2004; Cutter 2008). We made this choice to minimize biological differences other than ovariole numbers among taxa and to facilitate the detection of putative cause–effect relationships (here, dN/dS and ovariole number; Felsenstein 1985; Bromham et al. 1996; Whittle and Johnston 2003; Thomas et al. 2010; Symonds and Blomberg 2014). The close relatedness of species is more conducive to accurate alignments and retains a larger set of orthologous genes, including rapidly evolving genes, for study, than when studying more divergent species, which often skews toward the identification of fewer and more slowly evolving orthologous gene sets (*cf.* Stanley and Kulathinal 2016; Bubnell et al. 2022), and may exclude some rapidly evolving genes of interest.; (iv) each species has a whole-genome sequence available (Gramates et al. 2022); and (v) the dN and dS values among the species in this subgroup have substantially diverged, yet are also unsaturated in the frequency of substitutions, and thus are within the ideal range for dN/dS analysis (Castillo-Davis et al. 2004; Larracuente et al. 2008; Treangen and Rocha 2011) (for example, from M0 dN/dS values [that is, the single clade-wide measure of dN/dS; Stanley and Kulathinal 2016], we found the 95th percentile for M0 dN = 0.235 and M0 dS = 0.791 for the 9,232 genes that had orthologs in all five species and M0 values). In sum, this closely related taxonomic group has multiple benefits for the study of the evolution of ovariole-related genes.

Identification of Rapidly Evolving Ovariole-Related Genes for Follow-Up Study

To identify genes associated with ovariole numbers or functions for study, we focused on three recently available datasets from *D. melanogaster*. The first gene set we designate as the SIGNALC dataset, defined here as the signaling and connector genes (connectors identified by protein interaction networks) that were identified as affecting ovariole or egg numbers in a *hpo[RNAi]* and/or a *hpo[+]* background (Kumar et al. 2020). Among 463 signaling genes and additional connector genes studied, the authors reported 67 genes that affected ovariole number in a *hpo[RNAi]* background (named therein *hpo[RNAi]* Ovariole Number), 59 and 49 genes that affected egg laying in a *hpo[RNAi]* background (*hpo[RNAi]* Egg Laying) and a wild-type (*wt*) background (Egg Laying *[wt]*), respectively, and 17 connector genes that altered ovariole or egg laying phenotypes (and passed screening of $z > 1$; note that genes may belong to more than one category) (Kumar et al. 2020). The second is the BULKSG dataset, based on bulk RNA-seq data obtained from pooled larval ovarian somatic cells or GC from the early (72 h after egg laying [AEL]), mid (96 h AEL), and late (120 h AEL) TF developmental stages (Tarikere et al. 2022) and identified differentially expressed genes (*P*-values were from DeSeq2; Love et al. 2014). The third is the SINGLEC dataset (Slaidina, Gupta et al. 2020), a sc-RNA-seq dataset that provided expression data for each of the cell types of the *D. melanogaster* LL3 larval ovary (Fig. 1) (Slaidina, Gupta et al. 2020). The SINGLEC study assessed average standardized expression to identify differentially expressed genes among cell types (*P*-values from Seurat v.2; some genes were upregulated in more than one cell type using the criteria therein; Slaidina, Gupta et al. 2020).

The SIGNALC, BULKSG, and SINGLEC gene sets were screened for further study using their clade-wide M0 dN/dS values (Yang 2007) that reflects the rate of protein divergence and the potential types of selective pressures that may have affected a gene (Yang 2007, 1997). Values of dN/dS <1 suggest a history of purifying selection on protein sequences, = 1 infer neutral evolution, and >1 suggest a history of positive selection (Yang 2007, 1997); however, even when dN/dS <1 across an entire gene (Yang 2007), elevated dN/dS values in one gene relative to another suggest an enhanced degree of positive selection and/or neutral evolution (Buschiazzo et al. 2012; Ho and Smith 2016; Mitterboeck et al. 2017; Whittle et al. 2021; Yang 1998, 2007). We identified those ovariole-related genes with an M0 dN/dS value at least 1.5-fold (SIGNALC; lower cut-off due to conserved nature of signaling genes, see

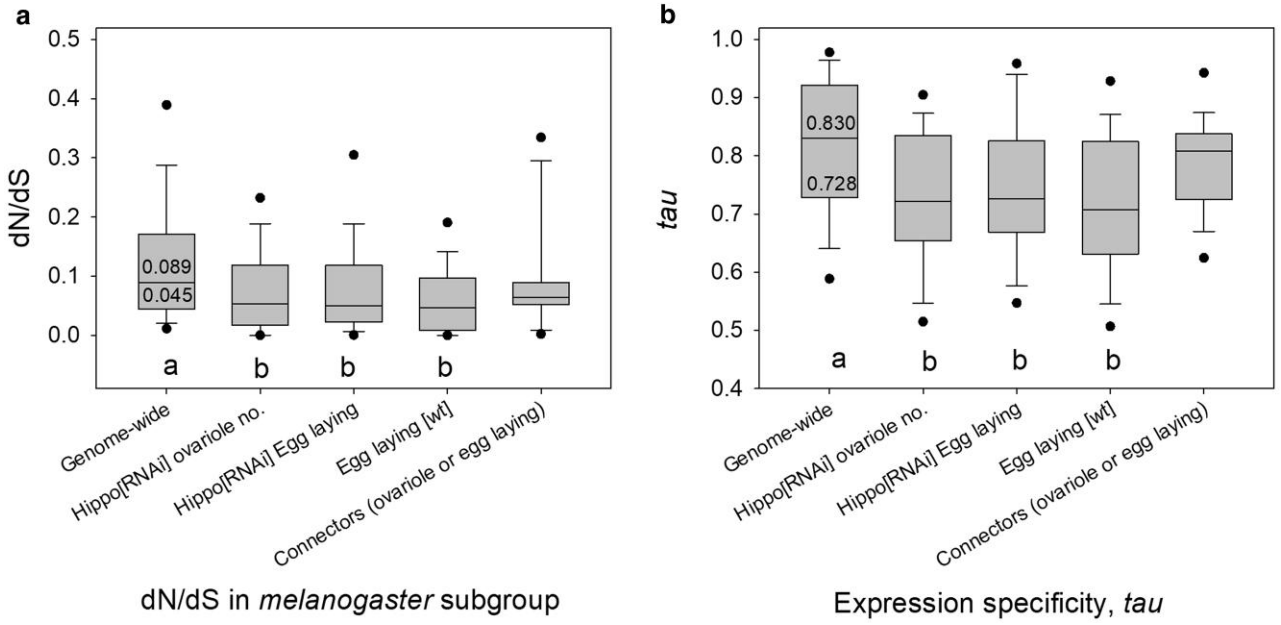

Fig. 3.—Box plots of a) M0 dN/dS of genes with five-species orthologs in the *melanogaster* subgroup for each of four groups of signaling/connector genes that affected ovariole/egg numbers using RNAi in *D. melanogaster* (Kumar, et al. 2020) and for the genome-wide values and b) *tau* for all genes in each of the four groups of ovariole number/egg laying affecting genes and the genome-wide values. Different letters (a. b) below bars indicate a statistically significant difference (MWU tests $P < 0.05$) between the genome-wide values and each group of genes. The median and 25th percentiles are shown for dN/dS and *tau* as reference points for the genome-wide values (i.e. across all 9,232 genes with known dN/dS and five-species orthologs).

Materials and Methods) or 2-fold (BULKSG and SINGLEC) higher than the genome-wide medians, and we then conducted a thorough follow-up analysis that included the M1 free-ratio species branch dN/dS (e.g. Dorus et al. 2004; Nadeau et al. 2007; Clark et al. 2009; Wlasiuk and Nachman 2010; Mensch et al. 2013; Borges et al. 2019; Kong et al. 2019; LaBella et al. 2021), branch-site tests of positive selection (Yang 2007; Zhang et al. 2005), *tau* (Yanai et al. 2005), and phylogenetic regressions (R-Core-Team 2022) (see Materials and Methods).

Some Signaling Pathway Genes that Regulate Ovariole Number Have Evolved Rapidly

We report that for the ovariole-related SIGNALC gene set (Kumar et al. 2020), which included signaling genes that affected ovariole number and/or egg laying, many genes exhibited very low M0 dN/dS (Mann-Whitney U (MWU) tests had $P < 0.05$ vs. the genome-wide values; Fig. 3a). This suggests a history of strong purifying selection on these highly conserved signaling genes, which may be partly due to their high pleiotropy, given that all of these signaling pathways play multiple roles in development and homeostasis (Kumar et al. 2020). Consistent with this hypothesis, the *tau* values for these genes were statistically significantly lower than the genome-wide values (MWU tests had $P < 0.05$; Fig. 3b), suggesting that broad expression breadth may have acted to slow molecular evolution (Otto 2004; Kim et al. 2007; Cui et al. 2009; Mank and Ellegren 2009; Meisel 2011; Assis et al. 2012; Masalia et al. 2017; Whittle et al. 2021).

Importantly, however, our main goal herein was to identify whether any ovariole-related SIGNALC genes evolved unusually rapidly and showed signs of evolvability that could underlie interspecies ovariole number divergence. As shown in supplementary table S2, Supplementary Material online, we indeed identified 27 SIGNALC genes that had elevated M0 dN/dS in at least one of the studied *Drosophila* taxon groups (≥1.5-fold higher than the genome-wide median; Table 1, supplementary table S2, Supplementary Material online, see also supplementary Text File S1 Results, Supplementary Material online, and Table 1 Notes for *Paris*). The signaling pathways and example functions of each of these genes are provided in supplementary table S3, Supplementary Material online: we found they were preferentially involved in developmental and cytoskeletal roles. Thus, it is apparent that while most of the ovariole number-related signaling genes evolved under strong purifying selection (Fig. 3a), a subset of them exhibited a high rate of amino acid sequence changes, well above the genome-wide median, in the *melanogaster* subgroup of *Drosophila*. This pattern shares similarities to the previous finding that while most *D. melanogaster* developmental genes expressed at the phylotypic stage of embryogenesis evolved under

Table 1

The gene-wide dN/dS per species branch values for each of the 27 signaling or connector genes (determined to be evolving rapidly in supplementary table S2, Supplementary Material online) in the five terminal species branches in the *melanogaster* subgroup of *Drosophila*

Fbgn ID	CG no.	Name	Symbol	Branch dN/dS					BR-S pos. sel. $P < 0.05$)	Gene phenotypic category in (Kumar, et al. 2020)
…	…	…	…	Dsim	Dsec	Dmel	Dyak	Dere	…	…
FBgn0011274	*CG6794*	*Dorsal-related immunity factor*	*Dif*	0.0001	0.5981	0.0001	**0.7233**	0.4146	Dyak	H-EL
FBgn0014020	*CG8416*	*Rho1*	*Rho1*	-	0.0001	-	0.0001	-	…	H-ON, H-EL, EL
FBgn0003612	*CG8068*	*Suppressor of variegation 2-10*	*Su(var)2-10*	**0.5723**	0.0001	0.5482	0.0662	0.0122	Dsim, Dmel	H-ON, H-EL, EL
FBgn0026379	*CG5671*	*Phosphatase and tensin homolog*	*Pten*	0.0001	0.1773	0.3122	0.1278	**0.5944**	Dere	H-ON
FBgn0000259	*CG15224*	*Casein kinase II beta subunit*	*CkIIbeta*	**0.6516**	0.2694	0.0001	0.0001	0.0001	Dsim	H-ON, H-EL, EL
FBgn0035213	*CG2199*	*CG2199*	*CG2199*	**1.0905**	0.404	0.3582	0.328	0.2765	…	Connector
FBgn0011642	*CG32018*	*Zyxin*	*Zyx*	0.3100	**>1**	0.2877	0.2668	0.3222	…	H-EL
FBgn0262614	*CG43140*	*polychaetoid*	*pyd*	0.0165	0.0341	**0.4745**	0.0168	0.0969	Dmel	H-ON
FBgn0036974	*CG5605*	*eukaryotic translation release factor 1*	*eRF1*	0.0001	0.1445	**0.3901**	0.0001	0.0697	Dmel	H-ON, H-EL, EL
FBgn0003984	*CG10491*	*vein*	*vn*	**0.4712**	0.2069	0.0841	0.2802	0.1511	Dyak	H-ON
FBgn0004858	*CG4220*	*Elbow B*	*elB*	0.0001	**0.6159**	0.0297	0.066	0.0617	Dsec	H-ON
FBgn0010825	*CG6964*	*Grunge*	*Gug*	**0.5467**	0.3674	0.0539	0.0469	0.0416	Dsim, Dsec	H-ON, H-EL, EL
FBgn0002174	*CG5504*	*CG5504*	*CG5504*	0.2527	**0.3135**	0.0423	0.0812	0.0937	Dsec	H-ON
FBgn0037218	*CG1107*	*auxilin*	*aux*	0.0648	0.1874	0.1639	**0.2738**	0.2413	Dere	H-EL, EL
FBgn0259176	*CG42281*	*bunched*	*bun*	0.0661	0.2569	0.1009	0.1814	**0.2716**	Dere	H-ON
FBgn0023540	*CG3630*	*CG3630*	*CG3630*	0.3585	**0.5938**	0.1247	0.239	0.1129	…	Connector
FBgn0261854	*CG42783*	*atypical protein kinase C*	*aPKC*	**0.1931**	0.0126	0.0001	0.0001	0.0855	Dsim	H-EL
FBgn0001169	*CG5460*	*Hairless*	*H*	0.1982	0.1646	0.1585	**0.2220**	0.1746	…	H-ON
FBgn0024291	*CG5216*	*Sirtuin 1*	*Sirt1*	0.0001	0.1876	**0.2589**	0.1113	0.071	Dmel	H-EL, EL
FBgn0030904	*CG5988*	*unpaired 2*	*upd2*	-	**0.4168**	0.0347	0.0793	0.1667	Dsec, Dere	H-ON, H-EL, EL
FBgn0020496	*CG7583*	*C-terminal binding protein*	*CtBP*	**0.5440**	0.0001	0.0697	0.0001	0.1103	Dsim	H-ON, H-EL, EL
FBgn0003607	*CG8409*	*Suppressor of variegation 205*	*Su(var)205*	0.1102	0.0001	**0.2107**	0.058	0.0634	…	Connector
FBgn0261592	*CG10944*	*Ribosomal protein S6*	*RpS6*	0.0001	**0.3052**	0.0179	0.0001	0.0001	Dsec	H-ON, H-EL, EL
FBgn0020386	*CG1210*	*Phosphoinositide-dependent kinase 1*	*Pdk1*	0.3558	**0.4589**	0.0996	0.0624	0.0490	Dsim, Dsec	H-ON
FBgn0002592	*CG6104*	*Enhancer of split m2, Bearded family member*	*E(spl)m2-BFM*	**0.5554**	0.2217	0.0197	0.1984	0.1469	Dyak	H-ON
FBgn0032006	*CG8222*	*PDGF- and VEGF-receptor related*	*Pvr*	0.0077	**0.2500**	0.0535	0.1614	0.2063	…	H-EL
FBgn0045035	*CG6535*	*telomere fusion*	*tefu*	0.0868	**0.1908**	0.1222	0.1051	0.1411	…	H-OV, EL

Branch-site positive selection (BR-S pos. sel.) analysis and cases with $P < 0.05$ are shown by species name (Dsim = *D. simulans*, Dsec = *D. sechellia*, Dmel = *D. melanogaster*, Dyak = *D. yakuba*, and Dere = *D. erecta*). The ovariole number/egg laying phenotypic categories defined in the RNAi experiments from (Kumar, et al. 2020) are shown here as: H-ON for *hpo[RNAi]* Ovariole Number, H-EL for *hpo[RNAi]* Egg Laying, and EL for the Egg Laying *[wt]*, and genes designated in that study as "connector genes" with observed phenotypes (on ovariole number or egg laying) are also shown.

Notes: a value of ">1" indicates that dN/dS > 1 and that PAML indicates the value of infinity, where dN > 0.001 and typically dS are approaching zero, and thus is simply denoted as dN/dS > 1, inferring positive selection. BR-S pos. sel.= branch-site positive selection. "-"indicates the dN and dS were each <0.001 and thus had too low divergence to determine dN/dS. The species branch per gene with the highest dN/dS is in **bold**. The connector gene *Paris* (FBgn0031610) was rapidly evolving in Dmel–Dsim but lacked high-confidence orthologs in all five species (supplementary table S2, Supplementary Material online). Genes that showed positive selection using McDonald and Kreitman (1991) tests of Dmel–Dsim included FBgn0026379 (*Pten*), FBgn0004858 (*elB*), FBgn0010825 (*Gug*), FBgn0261854 (*aPKC*), and FBgn0032006 (*Pvr*). One gene, *Zyx,* was not available for MK tests in the database (Murga-Moreno, et al. 2019).

strong purifying selection (low dN/dS), a subset of genes expressed at this stage exhibited a history of positive selection (Mensch et al. 2013).

Rapid and Adaptive Evolution of Specific Signaling Genes Coincides with Ovariole Number Evolution

To examine potential lineage-specific patterns of molecular evolution and pleiotropy of the 27 rapidly evolving ovariole-related genes, we assessed dN/dS per species branch (Table 1), branch-site positive selection (Table 1), and *tau* (supplementary table S3, Supplementary Material online). We found that these 27 genes showed marked differences in dN/dS values per gene among the five species terminal branches in the *melanogaster* subgroup (the distribution of dN/dS for all genome-wide genes per species branch is shown in box plots in supplementary fig. S1, Supplementary Material online). In addition, we observed branch-site positive selection in at least one species branch for 19 of the 27 genes (Table 1), which is consistent with potential high adaptability of these genes. Of particular note is the *D. sechellia* branch, as this species evolved a very low ovariole number (17 ovarioles per female, Fig. 2), only half that of its most closely related sister species *D. simulans* (33.9 ovarioles per female, Fig. 2), since diverging from their recent common ancestor. Among the five species terminal branches, the *D. sechellia* terminal branch had the highest dN/dS values for nine genes (Table 1), namely *Zyx*, *elB*, *CG5504*, *CG3630*, *upd2*, *RpS6*, *Pdk1*, *Pyr*, and *tefu*, with values ranging from 0.191 to >1. Further, five of these genes exhibited branch-site positive selection on amino acids in the *D. sechellia* branch (*elB, CG5504, unp2*, *RpS6, Pdk1*, branch-site $P < 0.05$ for all genes; Zhang et al. 2005; Yang 2007), explicitly showing a propensity for adaptive evolution in this species branch. In total, six of the 27 genes (22.2%) exhibited branch-site positive selection in the *D. sechellia* terminal branch. This was nearly double the genome-wide frequency for this species, which was 12.0% of 9,232 genes (one-tailed χ^2 $P = 0.05$). Thus, the *D. sechellia* lineage, with the lowest ovariole numbers (Fig. 2), has a dynamic molecular evolutionary history of ovariole number-regulating genes, consisting of rapid gene-wide evolution (dN/dS), combined with a pervasiveness of positive selection events on such genes in that species branch. The evolution of unusually low ovariole numbers in *D. sechellia,* which corresponded to gene sequence changes (relative to its close sister species) observed here, shares parallels to prior findings of a rapid shift from a "hairy" (with many larval cuticular bristles) phenotype in first-instar larvae in multiple *melanogaster* species (including *D. melanogaster*) to hairlessness within *D. sechellia*. In that case, the transition to hairlessness was shown to be caused by interspecies evolution at the *ovoD/shaven baby* locus (Sucena and Stern 2000), thus also demonstrating an association between gene changes and phenotype divergence within the *D. sechellia* lineage.

In *D. sechellia*'s sister species *D. simulans* (Fig. 2), eight genes had the highest dN/dS values in the *D. simulans* terminal branch (Table 1), five of which also exhibited statistically significant branch-site positive selection (*Su(var)2 to 10*, *CkIIbeta*, *Gug*, *aPKC*, *CtBP*, $P < 0.05$, Table 1). In total, six of the studied 27 SIGNALC genes (22.2%) presented branch-site positive selection in the *D. simulans* branch, which was more than 4-fold higher than the genome-wide frequency for the species (5.4%, χ^2 $P < 0.05$). In turn, four of 27 genes had the highest dN/dS in the *D. melanogaster* branch, and four genes had branch-site positive selection in *D. melanogaster* (14.8%), which was more than triple its genome-wide frequency (4.1%; χ^2 $P < 0.05$). *D. yakuba* and *D. erecta* had the highest dN/dS for three and two genes, respectively, and had branch-site positive selection in three and four genes, respectively (Table 1). In sum, for the *melanogaster* subgroup, all five species terminal branches showed signs of having the highest dN/dS values for at least two (*D. erecta*) and up to nine (*D. sechellia*) genes, exhibited signals of branch-site positive selection, and had particularly high rates of protein sequence divergence.

The patterns in Table 1 support the hypothesis that protein sequence changes, including adaptive changes, in these ovariole-related genes may underlie the genetic basis for the marked divergence in interspecies ovariole numbers (Fig. 2). For many of these genes, their known molecular and genetic mechanisms of action in tissue morphogenesis make them prime candidates for future analyses of how their diverged functions between species may have contributed to species-specific ovariole number evolution. For example, *Zyx* (*Zyxin*) is an actin cytoskeleton regulator and a signal transducer in the Hippo pathway, and mis-regulation of either actin cytoskeleton function (Li et al. 2003) or Hippo signaling function (Sarikaya and Extavour 2015; Kumar et al. 2020) during ovariole morphogenesis can alter ovariole number. We provide further discussion of some of these ovariole-related signaling genes in Table 1 in the supplementary Text File S1, Supplementary Material online.

Most of the Rapidly Evolving Signaling Genes were Differentially Expressed in Larval Ovary Cells

We identified genes whose high differential expression in the *D. melanogaster* larval ovary suggested a role in ovariole number regulation using the BULKSG RNA-seq datasets using pooled larval ovarian somatic versus pooled GC from different stages of TF formation (Tarikere et al. 2022). First, we asked whether the 27 rapidly evolving ovariole-related SIGNALC genes in Table 1 exhibited statistically significant differential expression between somatic and GC during TF formation (therein $P < 0.01$; Love et al. 2014; Tarikere et al. 2022). Remarkably, as shown in supplementary table

S4, Supplementary Material online, we report that 25 of the 27 rapidly evolving SIGNALC ovariole-related genes showed up- or downregulation in the soma (vs. GC; each cell type pooled across stages) or among the three different TF formation stages. Thus, this affirms that the SIGNALC genes in Table 1 that experimentally affected ovariole numbers or functions using RNAi (Kumar et al. 2020), and that showed signals of enhanced evolvability herein (Table 1), also exhibited differential expression in the larval somatic ovary cells, based on an independent approach of bulk RNA-seq (Tarikere et al. 2022). These two lines of evidence suggest that these genes are apt to have contributed toward the genetic basis of evolved ovariole number divergence.

Rapidly Evolving Genes are Highly Transcribed in the Larval Ovary Somatic Cells

We aimed to further identify any rapidly evolving genes that were highly differentially expressed in the larval ovarian soma during TF formation and thus potentially involved in the evolution of ovariole number using the BULKSG datasets. For this, we identified genes that were upregulated in the soma versus the GC, ranked them by $\log_2$fold upregulation, and, in that subset, screened for genes that were rapidly evolving in the *melanogaster* subgroup as compared to the genome-wide values (see Materials and Methods, M0 dN/dS > 0.20). The top ten genes matching these criteria are shown in Table 2, with the highest $\log_2$fold values ranging from 5.1 to 10.0, which includes the branch dN/dS, branch-site positive selection tests for each species of the *melanogaster* subgroup, and *tau* values (see supplementary Text File S1, Supplementary Material online for analysis of genes highly upregulated in GC, supplementary table S5, Supplementary Material online).

Remarkably, eight of the ten most highly upregulated and rapidly evolving somatic genes had extremely elevated *tau* values >0.90, and six had values above 0.94, indicating very narrow expression breadth (as compared to genome-wide values in supplementary fig. S2, Supplementary Material online). This low pleiotropy may facilitate their rapid evolution, via neutral evolution, and/or by adaptive sequence evolution (Otto 2004; Larracuente et al. 2008; Mank and Ellegren 2009). For the *D. sechellia* branch, five of the ten genes had the highest dN/dS in this species terminal branch, including *Ilp5 (insulin-like peptide 5*, dN/dS = 0.5843, discussed in supplementary Text File S1, Supplementary Material online) and four unnamed genes (CG identifiers only, *CG32581*, *CG31157*, *CG10232*, *CG30281*). Two of these, *CG31157* and *CG10232,* exhibited gene-wide positive selection with dN/dS values larger than 1, and the latter gene also had dN/dS >1 in *D. simulans* (Table 2). Further, *CG31904* exhibited branch-site positive selection in *D. sechellia* (Table 2). These patterns are consistent with a prevalent history of rapid protein evolution coupled with the ovariole number decline within the *D. sechellia* branch, as also observed for multiple SIGNALC genes (Table 1). Further, three of the ten genes also showed branch-site positive selection in *D. melanogaster*, and one displayed this pattern in *D. erecta* (Table 2), suggesting that many of these genes experienced a history of adaptive evolution across multiple lineages of the phylogeny.

TF Cells and SH Cells Express Rapidly Evolving Genes

The SINGLEC dataset was based on sc-RNA-seq data generated from the late third-instar *D. melanogaster* ovary (Slaidina, Gupta et al. 2020) and includes expression data

Table 2

Genes that were highly upregulated in the larval ovary somatic cells relative to germ cells when pooled across three larval stages (Tarikere, et al. 2022) and that exhibited rapid protein sequence divergence in the *melanogaster* subgroup (M0 dN/dS > 0.20)

Fbgn ID	$\log_2$fold change	Gene name	Gene symbol	M0 dN/dS	Branch dN/dS					BR-S pos. sel. $P < 0.05$	*tau*
…	…	…	…	…	Dsim	Dsec	Dmel	Dyak	Dere	…	…
FBgn0052581	10.012	*CG32581*	*CG32581*	0.3052	0.1647	**0.6899**	0.5668	0.2455	0.1672	Dmel	0.7378
FBgn0051157	9.389	*CG31157*	*CG31157*	0.2962	0.1163	**1.3228**	0.1967	0.3405	0.1777	…	0.9010
FBgn0039108	7.526	*CG10232*	*CG10232*	0.7202	>1	**2.0881**	0.4894	0.6514	0.5914	Dere	0.9260
FBgn0039598	7.217	*Aquarius*	*aqrs*	0.2305	0.1183	0.1097	0.1265	**0.3029**	0.1972	…	0.9946
FBgn0260479	5.373	*CG31904*	*CG31904*	0.3038	0.0001	0.4808	**0.6401**	0.0556	0.1363	Dsec, Dmel	0.9466
FBgn0044048	5.343	*Insulin-like peptide 5*	*Ilp5*	0.3776	0.2932	**0.5843**	0.0001	0.4907	0.5501	…	0.8485
FBgn0031900	5.308	*CG13786*	*CG13786*	0.2487	0.1709	0.2778	0.2748	0.2362	**0.3271**	…	0.9483
FBgn0050281	5.216	*CG30281*	*CG30281*	0.2155	0.4796	**0.613**	0.1951	0.1806	0.249	…	0.9820
FBgn0031646	5.146	*Snustorr snarlik*	*snsl*	0.2672	0.1571	0.0635	0.1566	0.2317	**0.585**	…	0.9408
FBgn0051815	5.070	*CG31815*	*CG31815*	0.3745	0.1725	0.3185	0.3421	**0.4465**	0.3695	Dmel	0.9519

The dN/dS per species terminal branch, branch-site positive selection ($P < 0.05$), and *tau* values are shown for each gene. The genes with the top 10 $\log_2$fold change values matching these criteria are shown.

Notes: The species branch per gene with the highest dN/dS is in **bold**. A name for FBgn0052581 as *suppression of retinal degeneration disease 1 upon overexpression 2* (*sordd2*) has been recently added/proposed at FlyBase. One gene, *CG10232*, showed positive selection using McDonald and Kreitman (1991) tests of Dmel–Dsim.

for all the cell types shown in Fig. 1 (the GC and eight somatic cell types, namely the CC, FSCP, IC, SHa cells, SHm cells, SW cells, anterior TF cells [TFa], and posterior TF cells [TFp]). Using hierarchical clustering of average standardized gene expression per gene, across all genes (supplementary fig. S3, Supplementary Material online), we found that the germ cells exhibited the most unique transcriptome of all studied cell types and formed an outgroup to all somatic cells. Among the somatic cells, the two types of TF cells, TFa and TFp, formed their own cluster, as did the two types of SH cells, SHm and SHa; each of these clusters was separate from all other somatic cell types (supplementary fig. S3, Supplementary Material online). The FSCP and SW cells had highly similar transcription profiles, as did the IC and CC. Thus, the TFs and SH cells had more distinctive transcriptomes than the other LL3 ovarian somatic cell types.

Rapidly Evolving Genes Identified in Both the BULKSG and SINGLEC Datasets

To identify genes with roles in specific ovarian cell types that were putatively involved in interspecies ovariole number divergence, we first extracted those SINGLEC genes that were upregulated in one cell type relative to all others ($P < 0.05$, analyzed in Seurat v. 2; genes could be upregulated in more than one cell type; Satija et al. 2015; Slaidina, Gupta et al. 2020) and that also had M0 dN/dS more than 2-fold above the genome-wide median (dN/dS > 0.20) within the *melanogaster* subgroup. We then compared this SINGLEC gene set to the 30 most highly differentially expressed and rapidly evolving genes identified from the somatic larval ovary cells at three different stages of development for TF formation (listed in supplementary table S6, Supplementary Material online, extracted from BULKSG dataset) and determined whether any genes were upregulated in both datasets. We identified five genes that matched these criteria: *Drip*, *CG3713*, *MtnA*, *vkg*, and *Col4a1* (Table 3). Among the various somatic cell types (Fig. 1), these genes were nearly exclusively upregulated in the TFs (TFa or TFp, or both) and/or the SHm cells. We note that *vkg* and *Col4a1* play roles in basement membrane formation (Yasothornsrikul et al. 1997; Kiss et al. 2019) and that SHm cells lay the membrane that separates the TFs for ovariole development (King 1970; Slaidina, Gupta et al. 2020). Given the crucial roles of these cell types in determining ovariole number (King, Aggarwal et al. 1968, Sarikaya and Extavour 2015), the rapid evolution of these five genes may partially underlie ovariole number divergence between species (King et al. 1968; Sarikaya and Extavour 2015) in the *melanogaster* subgroup (Table 3).

In terms of molecular evolution per terminal species branch, the five genes in Table 3 exhibited a striking propensity for adaptive evolution. Four of the five genes showed a gene-wide level of positive selection (terminal

Table 3

Genes with rapid divergence (M0 dN/dS > 0.20) and that were highly upregulated at one stage of the larval ovary somatic cells (vs. the others; three stages, namely early, mid, and late, among the top 30 most upregulated genes) in Dmel using BULKSG data (Tarikere, et al. 2022) and that also exhibited upregulation in at least one cell type (vs. all others) using SINGLEC data among the nine studied LL3 ovary cell types (Slaidina, Gupta et al. 2020)

Fbgn ID	Gene	BULKSG Up		SINGLEC Up (Seurat $P < 0.05$)	M0 dN/dS	Branch dN/dS				Branch-site positive selection $P < 0.05$				*tau*	Example of key function
		Stage up	log_2fold change			Dsim	Dsec	Dmel	Dyak	Dere	Dsim	Dsec	Dmel	Dyak	Dere		
…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…
FBgn0015872	*Drip*	Late	7.798	TFa, TFb	0.2734	0.6248	>1	0.4140	0.3750	0.1312	yes	…	yes	yes	…	0.9786	Membrane
FBgn0040343	*CG3713*	Late	5.951	TFa	0.2636	**>1**	0.0001	0.8226	0.1501	0.3225	…	…	…	…	…	0.9146	Uncharacterized
FBgn0002868	*MtnA*	Early	4.380	TFp, CC	0.6883	-	>1	**>1**	**>1**	0.1265	…	…	…	…	…	0.8753	Response to metal ion
FBgn0016075	*vkg*	Late	4.200	TFa, TFp, SHm, CC	0.3860	0.3038	**0.7557**	0.2965	0.3510	0.5572	…	yes	…	yes	yes	0.9037	Basement membrane
FBgn0000299	*Col4a1*	Late	3.875	TFa, TFp, SHm, CC	0.4065	0.3034	**1.2519**	0.2072	0.4728	0.6879	…	yes	…	yes	yes	0.8887	Basement membrane

Shown are the dN/dS per species branch, the presence of branch-site positive selection ($P < 0.05$), the *tau* values, and an example of key functionality as described in DAVID (Huang et al. 2009). "Stage up" indicates the larval ovary stage where the gene was upregulated ($P < 0.05$). SINGLEC up indicates the cell type(s) with upregulation.

Notes: The cell types with upregulation are shown by the following abbreviations TFa = terminal filaments anterior, TFp = terminal filaments posterior, SHm = sheath cells migrating, and CC = cap cells. "-" indicates the dN and dS were each <0.001 and thus have too little divergence to be able to determine dN/dS. The species branch with the highest dN/dS is in **bold**. Genes that showed positive selection using McDonald and Kreitman (1991) tests of Dmel–Dsim included FBgn0016075 (*vkg*) and FBgn0000299 (*Col4a1*).

branch dN/dS values >1) in at least one species branch (Table 3). Moreover, *Drip*, *vkg*, and *Col4a1* each exhibited branch-site positive selection in three different species branches ($P < 0.05$), suggesting a profound history of adaptive changes across multiple lineages. In addition, McDonald and Kreitman (1991) tests also showed positive selection for *vkg* and *Col4a1* ($P < 0.05$, Table 3 Notes). All five genes exhibited *tau* values above 0.875 with *Drip* having a value of 0.979, suggesting especially high expression specificity (see Materials and Methods, supplementary fig. S2, Supplementary Material online), which may facilitate the observed adaptive evolution of the protein sequences (Otto 2004; Mank and Ellegren 2009; Whittle et al. 2021). In sum, these five genes were identified from two distinct expression datasets (Slaidina, Gupta et al. 2020; Tarikere et al. 2022), were upregulated in two of the most crucial cell types for ovariole number determination, namely TFs and SH cells (supplementary table S6, Supplementary Material online, Table 3), and exhibited rapid protein changes, positive selection, and narrow expression breadth (Table 3). Thus, multiple lines of evidence point toward these genes as having a central role in the interspecies divergence of ovariole number.

Genes Upregulated in TF and SH Cells Frequently Display Branch-Site Positive Selection

We assessed the frequency of genes that exhibited branch-site positive selection ($P < 0.05$) per species terminal branch for the rapidly evolving genes that were upregulated in each of the nine cell types in the SINGLEC dataset ($P < 0.05$). The results for *D. simulans*, *D. sechellia*, and *D. melanogaster* (a very closely related species group with substantial differences in ovariole numbers; Fig. 2) are shown in Fig. 4 and for all five species in supplementary fig. S4, Supplementary Material online. The genes with the highest percent branch-site positive selection were those upregulated in the SH and TF cells (Fig. 4; the TF and SH genes are listed in supplementary table S7, Supplementary Material online). Specifically, positive selection was most commonly observed for genes up-regulated in the SHm cells for the *D. sechellia* branch (45%), from the TFa (34.1%) and TFp (36.7%) cells in the *D. sechellia* branch, and for SHa cells in the *D. sechellia* (33.33%) and *D. simulans* (33.33%) branches (all values were statistically significantly higher than the genome-wide percentages of genes with branch-site positive selection per species, which were 5.4% for *D. simulans* and 12.0% for *D. sechellia*; χ^2 $P < 0.05$, Fig. 4). Thus, the most important somatic cell types for ovariole number determination (TF and SH cells) (King et al. 1968; Godt and Laski 1995; Sarikaya et al. 2012; Sarikaya and Extavour 2015; Slaidina, Gupta et al. 2020) are also those in which highly upregulated genes most commonly exhibited branch-site positive selection, particularly in *D. sechellia*.

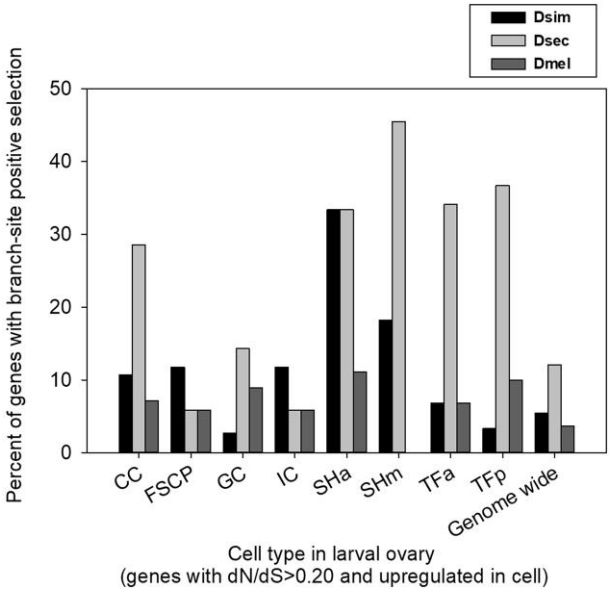

Fig. 4.—The percentage of the genes that were both upregulated in a particular cell type and rapidly evolving in the *melanogaster* subgroup (M0 dN/dS > 0.20) that exhibited branch-site positive selection in the *D. simulans* (Dsim), *D. sechellia* (Dsec), and *D. melanogaster* (Dmel) branches ($P < 0.05$). The number of genes per category was as follows: CC (28), FSCP (17), GC (112), IC (17), SHa (9), SHm (11), TFa (44), and TFp (30). SW cells were excluded as too few genes were rapidly evolving for study (SW: 4). Note that a gene could be upregulated in more than one cell type. The genome-wide values are for all genes with five-species orthologs in the *melanogaster* subgroup.

The genes identified above as highly expressed in TF and SH cells could also be highly expressed in additional cell types (Slaidina, Gupta et al. 2020). Indeed, on average we found that differentially expressed genes were upregulated in 1.9 ± 0.02 cell types. Thus, for additional stringency we isolated the subset of rapidly evolving genes (with M0 dN/dS > 0.20) that were upregulated in only one cell type. While most somatic cell types had very few genes matching this stringent criterion ($N \leq 4$ per cell type), by pooling the two types of SH cells (SHa and/or SHm) and TF cells (TFa and/or TFb) we found 8 and 26 such genes in these cell types, respectively (provided in supplementary table S7 Notes, Supplementary Material online). We found that *D. simulans*, *D. sechellia*, and *D. melanogaster* showed branch-site positive selection in 25.0%, 25.0%, and 0% of these genes, respectively, for SH cells and in 11.5%, 23.1%, and 7.7% of these genes, respectively, for TF cells. These values were well above the genome-wide frequency for *D. sechellia* and *D. simulans* (although tests were conservative due to sample size, χ^2 *P* values for SH for *D. simulans* = 0.047 and TF for *D. sechellia* = 0.077 relative to the genome-wide values). In sum, interpreting the results in Fig. 4 conservatively, we observe that upregulation of a

gene in TF or SH cells is correlated with enhanced rates of positive selection in the *D. sechellia* and/or *D. simulans* lineages, regardless of whether the genes were also upregulated in another cell type (Fig. 4; supplementary table S7, Supplementary Material online).

While we focused on the three-species clade in Fig. 4, the results for all five *melanogaster* subgroup species are provided in supplementary fig. S4, Supplementary Material online. Of particular note, those results showed that 45.5% of the genes that were upregulated in the SHm cells also exhibited positive selection in the *D. yakuba* and in the *D. erecta* terminal branches (similar to *D. sechellia* in Fig. 4, supplementary table S7, Supplementary Material online). This suggests a history of branch-site positive selection for genes expressed in the SHm cells across outgroup branches of the phylogeny, potentially partly contributing to the divergence in ovariole numbers or functions in the two outgroup species from the three ingroup species (Fig. 2).

Functional Predictions for Upregulated TF and SH Genes

The studied molecular evolutionary parameters for all rapidly evolving genes studied in Fig. 4 that were upregulated in SHa, SHm, TFa, and TFp are provided in supplementary table S7, Supplementary Material online. Analysis of GO-predicted functions using DAVID (Huang et al. 2009) showed that the genes expressed in SHa and SHm cells, such as *Jupiter* and *Timp* (supplementary table S7, Supplementary Material online), were preferentially involved in microtubule formation and basement membranes (Huang et al. 2009), consistent with roles in TF formation (Slaidina, Gupta et al. 2020). The highly upregulated and rapidly evolving TF genes in Fig. 4 and supplementary table S7, Supplementary Material online were more than 3-fold more common than the SH cell genes and thus allowed us to perform functional clustering (Huang et al. 2009). As shown in supplementary table S8, Supplementary Material online, the TF genes were preferentially associated with extracellular matrix (20.5% and 23.3% of genes from TFa and TFp, respectively) and basement membranes (6.8% and 10%), and 40% of genes from TFp were an integral component of membranes.

The TF and SH cells types in Fig. 4 have been experimentally shown to regulate the formation and number of ovarioles in *D. melanogaster* larvae (King et al. 1968; King 1970; Godt and Laski 1995; Dansereau and Lasko 2008; Sarikaya et al. 2012; Sarikaya and Extavour 2015; Slaidina, Gupta et al. 2020). Thus, that fact the genes (in Fig. 4) were highly expressed in these specific cell types, exhibited rapid sequence evolution and had signals of adaptive evolution (Fig. 4, supplementary table S7, Supplementary Material online), suggests that they have the potential to directly cause the interspecies shifts in ovariole numbers (Fig. 2). In turn, it may also be the case that the protein sequence changes observed in some of these genes may be in response to evolved shifts in ovariole numbers (potentially mediated by other ovariole-involved genes identified herein) and thus that the adaptive gene changes that we report here reflect responses to the physiological intracellular changes in TFs and SH cells needed to support ovariole number changes.

Molecular Evolutionary Rates of Key Genes Predict Ovariole Number

Finally, we conducted follow-up assessments of the main genes identified throughout our study that showed signs of high evolvability, positive selection, and involvement in *Drosophila* ovariole number divergence, to determine to what extent the molecular evolutionary characteristics of these genes were predictive of ovariole numbers in the context of *Drosophila* phylogeny. Specifically, for all genes identified from SIGNALC ($N = 27$; Table 1), from BULKSG ($N = 10$; Table 2), and from BULKSG and SINGLEC combined ($N = 5$; Table 3), we conducted a phylogenetic generalized least square (PGLS) assessment of the relationship between ovariole number and the dN/dS values for the 41 of these 42 genes that were testable (*MtnA* was untestable due to infinity dN/dS [near zero dS, dN > 0] in several branches; Table 4; a summary of McDonald and Kreitman (1991) test values for all genes is shown in supplementary table S9, Supplementary Material online). We found that 17 of the 41 testable genes (41.5%) showed a statistically significant relationship between ovariole number and dN/dS value (Table 4; $P < 0.05$, *CG3630* had $P < 0.07$ and was noted in the list), indicating that dN/dS values of these genes can be a predictive factor for ovariole number per species. This further demonstrates the high effectiveness of utilizing protein sequence analysis to identify genes putatively involved in the evolution of phenotypes, similar to suggestions for other diverse traits across multiple taxa (Dorus et al. 2004; Nadeau et al. 2007; Ramm et al. 2008; Wlasiuk and Nachman 2010; Luke et al. 2014; Corso et al. 2016; Chebbo et al. 2021).

Supplementary Analysis of a Three-Species Clade of Hawaiian *Drosophila*

While we focused on the *melanogaster* subgroup for our core analyses, as a supplementary assessment, we considered a three-species clade of Hawaiian *Drosophila* that matched our strict criteria for study (very closely related species, genome-wide data, known [and variable] ovariole numbers). We note, however, that these species are relatively distantly related to *D. melanogaster*, the species used to identify ovariole-involved genes on the basis of function and/or expression (the SIGNALC, BULKSG, and SINGLESC datasets). Hawaiian *Drosophila* are paraphyletic to the *melanogaster* subgroup (Suvorov et al. 2022), and estimates of divergence

Table 4

PGLS analysis of the relationship between ovariole number and dN/dS for genes putatively involved in ovariole number evolution from Tables 1, 3, and 4 (42 genes total)

FBgn ID	Symbol	Dataset	Table with gene	PGLS *P*-value	Intercept	Slope	Predicted ovariole no. Under PGLS model				
…	…	…	…	…	…	…	Dsim	Dsec	Dmel	Dyak	Dere
FBgn0011274	*Dif*	SIGNALC	Table 1	0.0189	38.0298	−25.8738	38.03	22.55	38.03	19.32	27.30
FBgn0003612	*Su(var)2-10*	SIGNALC	Table 1	0.0115	22.0048	28.8688	38.53	22.01	37.83	23.92	22.36
FBgn0011642	*Zyx*	SIGNALC	Table 1	0.0205	35.9756	−14.2358	31.56	14.62	31.88	32.18	31.39
FBgn0004858	*elB*	SIGNALC	Table 1	0.0170	32.7685	−28.2679	32.77	15.36	31.93	30.90	31.02
FBgn0259176	*bun*	SIGNALC	Table 1	0.0316	43.5717	−83.9484	38.02	22.01	35.10	28.34	20.77
FBgn0023540	*CG3630*[a]	SIGNALC	Table 1	0.0689	41.6675	−46.7838	24.90	13.89	35.83	30.49	36.39
FBgn0030904	*upd2*	SIGNALC	Table 1	0.0143	34.3707	−42.2274	34.37	16.77	32.91	31.02	27.33
FBgn0003607	*Su(var)205*	SIGNALC	Table 1	0.0092	18.0230	120.0788	31.26	18.04	43.32	24.99	25.64
FBgn0261592	*RpS6*	SIGNALC	Table 1	0.0261	31.9450	−55.4869	31.94	15.01	30.95	31.94	31.94
FBgn0032006	*Pvr*	SIGNALC	Table 1	0.0162	38.4848	−71.3851	37.94	20.64	34.67	26.96	23.76
FBgn0045035	*tefu*	SIGNALC	Table 1	0.0520	49.0222	−158.1686	35.29	18.84	29.69	32.40	26.70
FBgn0051157	*CG31157*	BULKSG	Table 2	0.0175	34.6446	−14.4238	32.97	15.56	31.81	29.73	32.08
FBgn0044048	*Ilp5*	BULKSG	Table 2	0.0225	44.4030	−40.6225	32.49	20.67	44.40	24.47	22.06
FBgn0015872	*Drip*	BULKSG and SINGLEC	Table 3	0.0474	38.3688	−16.6128	27.99	13.45	31.49	32.14	36.19
FBgn0040343	*CG3713*	BULKSG and SINGLEC	Table 3	0.0470	22.8789	10.9136	39.25	22.88	31.86	24.52	26.40
FBgn0016075	*vkg*	BULKSG and SINGLEC	Table 3	0.0171	45.3114	−37.1647	34.02	17.23	34.29	32.27	24.60
FBgn0000299	*Col4a1*	BULKSG and SINGLEC	Table 3	0.0053	39.2022	−18.3941	33.62	16.17	35.39	30.51	26.55

The 17 genes that showed a relationship using PGLS are shown ($P < 0.05$), and the intercept, the slope, and the predicted ovariole numbers using the model are included. In addition, the dataset that each gene was identified from and the table it is presented in are provided.

[a]Included as close to cut-off and $P = 0.069$. The phylogeny is shown in Fig. 2.

time since the last common ancestor of extant species from the two taxon groups exceed 60 Mya (Tamura et al. 2004; Goldman-Huertas et al. 2015). We chose the species *Drosophila sproati* (mean 65.6 ovarioles), *Drosophila murphyi* (mean 41.6 ovarioles), and *Drosophila grimshawi* (mean 47.8 ovarioles) for study, with a phylogeny of: (*D. sproati*, *D. murphyi*), *D. grimshawi* shown in supplementary fig. S5, Supplementary Material online, from Kim et al. (2021) and Suvorov et al. (2022) and ovariole numbers from Starmer et al. (2003), Sarikaya et al. (2012), and Sarikaya et al. (2019). For dN/dS analysis, we focused on the ovariole-involved SIGNALC genes identified in Table 1, as these signaling proteins are functionally confirmed to regulate ovariole number (Srivastava et al. 2010; Kumar et al. 2020). Thus, among the studied gene sets (SIGNALC, BULKSG, SINGLEC), we considered them the most appropriate for dN/dS analysis in a divergent group. We found that 21 of the 27 rapidly evolving ovariole-related genes in Table 1, which were identified from study of the *melanogaster* subgroup, had a high-confidence three-species orthologous gene set in the Hawaiian *Drosophila* clade (supplementary table S10, Supplementary Material online). Our evaluation of branch dN/dS values revealed that ten of the 21 genes evolved especially rapidly, with dN/dS > 0.33 in at least one species terminal branch in the Hawaiian clade, which was more than 2-fold higher than the genome-wide dN/dS values for the species under study (13 of 21 genes evolved rapidly using a criterion of 1.5-fold higher than the genome-wide medians; genome-wide dN/dS median values = 0.152, 0.164, and 0.160 for *D. murphyi*, *D. sproati*, and *D. grimshawi*, respectively; supplementary table S10 and supplementary Text File S1, Supplementary Material online). Moreover, *D. sproati*, the ingroup species with highest ovariole number per female of all three Hawaiian species (supplementary fig. S5, Supplementary Material online), had eight of the ten genes with dN/dS > 0.33 (supplementary table S10, Supplementary Material online). The ten most rapidly evolving genes included *upd2*, *CG2199*, *vn*, *elB*, *bun*, *CG3630*, *aPKC*, *H*, *Su(var)205*, and *E(spl)m2-BFM*, six of which also exhibited branch-site positive selection in at least one branch. For *upd2*, we observed branch (dN/dS > 1) and branch-site positive selection ($P < 0.05$) for all three species branches (supplementary table S10, Supplementary Material online), suggesting that it may have a putative role in ovariole number divergence in all three species. Nonetheless, it is notable that in supplementary table S10, Supplementary Material online, eight of the 21 genes had branch dN/dS below the aforementioned thresholds (were not 2-fold or 1.5-fold higher than the genome median) in all three Hawaiian species branches (supplementary table S10, Supplementary Material online). This suggests that while these genes may be involved in ovariole functions in those taxa (as they are in *D. melanogaster*; Kumar et al. 2020), their protein sequence divergence may be less apt to shape interspecies shifts in ovariole numbers in these Hawaiian *Drosophila* species (supplementary table S10, Supplementary Material online). Together, the data

suggest that a substantial number of the rapidly evolving ovariole-involved genes in Table 1 also evolved very rapidly in the Hawaiian clade and thus may have possibly contributed to its interspecies divergence in ovariole numbers.

We also examined the Hawaiian *Drosophila* species orthologs of some of the rapidly and adaptively evolving genes in the *melanogaster* subgroup, which we identified from the SINGLEC transcription dataset (Slaidina, Gupta et al. 2020) shown in Fig. 4 (and supplementary fig. S4, Supplementary Material online, *N* values per cell type shown therein; the TF and SH cell genes are in supplementary table S7, Supplementary Material online, which include certain genes from BULKSG in Table 3). We hypothesized that for these genes, identified as candidate ovariole number regulators based on *D. melanogaster* expression profiles alone, it might be harder to confidently assume conservation of function in ovariole number regulation in a clade as distantly related as the Hawaiian *Drosophila* (Ranz et al. 2003; Whittle and Extavour 2019). We therefore adopted a prudent approach, based on evaluation of the rate of high-confidence ortholog detection in the Hawaiian group (see Methods and Results in supplementary Text File S1, Supplementary Material online). As shown in supplementary fig. S6, Supplementary Material online, we found that genes in the TF and SH cells (Fig. 4, supplementary fig. S4, Supplementary Material online) had the fewest high-confidence Hawaiian orthologous gene sets, as compared to genes highly expressed in the other ovarian cell types (orthologs were defined as having an ortholog found in all three Hawaiian species and between *D. melanogaster* and *D. grimshawi* for gene identification). Specifically, genes upregulated in the SHa cells and those in the TFp cells (supplementary fig. S6, Supplementary Material online) each had 66.6% of genes with an orthologous Hawaiian three-species orthologous gene set. In contrast, genes upregulated in CC had 85.7% and FSCP and IC each had 82.4% (supplementary fig. S6, Supplementary Material online). We speculate that genes expressed in the TF and SH cells may have evolved at a relatively higher rate (Fig. 4, supplementary fig. S4 and table S7, Supplementary Material online) than those expressed in other ovarian cell types, making orthologs more frequently unrecognizable between *D. melanogaster* and the Hawaiian clade and/or among the three species in the Hawaiian clade (Tautz and Domazet-Loso 2011; Tautz et al. 2013) (discussed further in Results in supplementary Text File S1, Supplementary Material online). This rapid evolution could potentially be due to adaptive sequence changes associated with ovariole number divergence in the genus (Fig. 4). It is also possible that there has been a greater propensity of genes directly involved in ovariole formation (TF and SH cells) to undergo gains and/or losses over evolutionary time (Tautz and Domazet-Loso 2011; Tautz et al. 2013) than genes involved in regulating the other ovarian cell types. While our central focus herein was on the interspecies divergence of ovariole number and protein sequences of orthologous genes within the very closely related *D. melanogaster* subgroup (Tables 1 to 4, Fig. 4), these supplementary analyses in a Hawaiian clade provide insights into the dynamics that may contribute to ovariole number divergence over extended time scales.

Discussion

While insects exhibit a diverse number of ovarioles, including across two orders of magnitude in the genus *Drosophila* alone (Hodin and Riddiford 2000; Starmer et al. 2003; Markow et al. 2009; Sarikaya et al. 2019; Church et al. 2021), little has been known about the genetic basis of rapid interspecies divergence of this fundamental female reproductive trait. Here, we directly tackled this issue by comprehensively determining a priori genes with experimental and/or transcriptional evidence for roles in determining ovariole numbers or functions in *D. melanogaster* (Kumar et al. 2020; Slaidina, Gupta et al. 2020; Tarikere et al. 2022) and then assessing their molecular evolutionary characteristics within very closely related species in the *melanogaster* subgroup. The results revealed a highly evolvable set of ovariole-related genes that exhibited high gene-wide dN/dS and/or branch-site positive selection in patterns consistent with a role in the evolution of ovariole number divergence (Tables 1 to 4, supplementary table S7, Supplementary Material online). Moreover, PGLS analyses supported a predictive relationship between ovariole number per species and dN/dS for many of the identified rapidly evolving ovariole-related genes (Table 4). From these collective results, we propose that the rapid interspecies ovariole number divergence in *Drosophila* (Fig. 2) has been facilitated by a group of highly evolvable genes with ovariole-related functions (42 identified and of focus herein; Kumar et al. 2020; Slaidina, Gupta et al. 2020; Tarikere et al. 2022) that exhibit a propensity for rapid evolution (gene-wide dN/dS) and adaptive protein sequence changes (Tables 1 to 3, supplementary table S7, Supplementary Material online, Fig. 4, supplementary fig. S4, Supplementary Material online). This hypothesis is further supported by the fact that all of the ovariole-related genes revealed herein have been explicitly demonstrated to regulate ovariole number (Kumar et al. 2020) and/or are highly and/or exclusively expressed in somatic ovarian cells whose behavior determines ovariole number (King et al. 1968; King 1970; Sarikaya et al. 2012; 2019; Slaidina, Gupta et al. 2020; Tarikere et al. 2022).

Evolvability of Ovariole-Related Genes and *tau*

The evolvability, defined here as the propensity of traits or gene sequences to diverge (Wagner and Zhang 2011; Cutter and Bundus 2020), including adaptive evolution, for the ovariole-related genes identified herein for the *melanogaster* subgroup (Tables 1 to 3; and for the rapidly evolving ovariole genes for Hawaiian *Drosophila*, supplementary table S10,

Supplementary Material online), may potentially reflect fitness advantages of the fixed ovariole-related mutations and/or may have been influenced by relaxed purifying selection. Previous studies have found that genes with high values of *tau* (Yanai et al. 2005), which suggests low pleiotropy (Mank and Ellegren 2009; Meisel 2011; Dean and Mank 2016), may exhibit relaxed purifying selection, thereby allowing both elevated neutral protein sequence changes (and thus elevated dN/dS) and greater potential for adaptive evolution (Otto 2004; Larracuente et al. 2008; Mank et al. 2008; Mank and Ellegren 2009; Meisel 2011; Whittle et al. 2021). Consistent with this pattern, we found that many of the rapidly evolving ovariole-associated genes, including those with explicit evidence of adaptive evolution from gene-wide dN/dS values larger than 1 or from branch-site positive selection tests ($P < 0.05$), also exhibited relatively high *tau* (Tables 2 and 3, supplementary table S3, Supplementary Material online).

Furthermore, with respect to pleiotropy, we found that high *tau* (low pleiotropy) was nearly universal in the rapidly and adaptively evolving ovariole-related genes identified from the BULKSG and SINGLEC datasets (Tables 2 and 3), which are expressed in the larval cells that give rise to ovarioles (*tau* ≥0.85, and most had values >0.90). The one exception to this had *tau* = 0.73 (*CG32581*) (Table 2). Thus, low pleiotropy is associated with high evolvability, and enhanced adaptive potential, in those gene sets. Nonetheless, it is notable that the rapidly evolving ovariole-related signaling genes (Table 1), which are involved in core cellular pathways (supplementary table S3, Supplementary Material online), included some genes with lower *tau* values (*tau* values ranged between 0.57 and 0.96, supplementary table S3, Supplementary Material online; see also Fig. 3B). Thus, it is evident that a subset of the rapidly evolving signaling genes had relatively wide expression breadth (see, for example, those below the median in table <0.72, supplementary table S3, Supplementary Material online). It may be speculated that while wide expression breadth may typically slow protein sequence evolution (Mank and Ellegren 2009; Meisel 2011; Assis et al. 2012; Whittle et al. 2021), these are tendencies rather than laws and thus have exceptions. Given that the signaling genes in Table 1 have been shown to play key roles in regulating ovariole numbers and functions (Kumar et al. 2020), they are apt to directly affect reproductive success and fitness. It may therefore be speculated that highly beneficial mutations in these particular genes (Table 1, supplementary table S3, Supplementary Material online) may be prone to reach fixation, even under wide expression breadth, given their roles in female reproductive output. While positive selection for broadly transcribed signaling genes could arise due to a gene's function in other nonovarian/ovariole tissues, which could then lead to the appearance of adaption from its role in ovarioles, a fitness benefit strong enough to override high pleiotropy appears less likely for nonsexual gene functions. We therefore suggest that the rapid and adaptive evolution of the signaling genes with low *tau* is likely best explained by their reproductive roles (Kumar et al. 2020). Nevertheless, irrespective of the roles of pleiotropy, the rapid protein sequence evolution and positive selection observed for the ovariole-related genes (Tables 1 to 3, Fig. 4) suggest a pervasive history of adaptive evolution events, which may have arisen from natural selection for adaption to changes in environment or oviposition substrates (Jagadeeshan and Singh 2007). It is also possible that these evolved changes may have resulted from sexual selection, given the dynamic sexual behaviors of *Drosophila*, as described below.

Putative Roles of Sexual Selection on Ovariole Number Evolution

Sexual selection may contribute to the adaptive evolution of reproductive characteristics and genes in animals (Swanson and Vacquier 2002; Clark et al. 2009), including in *Drosophila* (Civetta and Singh 1998; Swanson et al. 2004; Proschel et al. 2006). Thus, one possibility is that this phenomenon may shape the evolution of ovariole-related genes observed herein (Tables 1 to 3, Fig. 4). Different species of *Drosophila* exhibit wide variation in their reproductive behaviors (Markow and O'Grady 2005), and examples of sexual selection in the genus include intrasexual selection from sperm competition (Singh et al. 2002; Singh and Singh 2014) and male–male (Singh and Singh 2014) and female–female competition (Bath et al. 2018). In addition, there is evidence of intersexual selection including female- and male-mate choice (Friberg and Arnqvist 2003; LeVasseur-Viens et al. 2015). In the latter case, if males favor larger females, a choice that may correlate with female fecundity in species where body size correlates positively with ovariole number (Bonduriansky 2001; Byrne and Rice 2006; Sinclair et al. 2021), then this could result in positive selection on amino acid changes favoring increased ovariole numbers. Moreover, *Drosophila* exhibits sexual antagonism, which could also potentially shape female (and male) reproductive characteristics and their underlying genes (Arnqvist and Rock 1995; Rice 1996; Swanson et al. 2004; Innocenti and Morrow 2010). For example, in *D. melanogaster*, some male reproductive traits and behaviors (e.g. seminal fluid toxicity, aggressive male remating behaviors) may be harmful to female reproduction and/or survival (Civetta and Clark 2000; Chapman et al. 2001; Sirot et al. 2014). Some studies have suggested that this could prompt female adaptive responses and give rise to adaptive changes in the *D. melanogaster* ovaries or eggs and in the protein sequences of genes expressed in the ovaries (Civetta and Clark 2000; Jagadeeshan and Singh 2005; Sirot et al. 2014). If this phenomenon also occurs across other members of the *melanogaster* subgroup, it may contribute to positive selection on ovariole numbers and thus on ovariole genes observed here. Significantly, sexual selection may affect

reproductive phenotypes and genes (Swanson and Vacquier 2002; Proschel et al. 2006) in a polygenic manner (Lande 1981; Coyne and Charlesworth 1997; Singh et al. 2001; Markow and O'Grady 2005; Singh and Singh 2014), which is relevant to ovariole number evolution as this is a highly polygenic trait (Coyne et al. 1991; Wayne and McIntyre 2002; Bergland et al. 2008; Green and Extavour 2012; Sarikaya and Extavour 2015; Lobell et al. 2017; Kumar et al. 2020).

Neutral Evolution and Ovariole Number

While we propose that our results could suggest an important role for adaptive evolution in ovariole-related genes in the interspecies divergence of ovariole numbers, it is worthwhile to consider the potential, and possibly complementary, roles of neutral evolution. Relaxed purifying selection in itself may lead to accelerated evolution and protein sequence changes (Kimura 1983; Mank and Ellegren 2009; Gossmann et al. 2012) and to an elevated gene-wide dN/dS in a particular branch. Thus, it may be possible that some selectively neutral amino acids in ovariole-related genes were fixed via random genetic drift and affected ovariole numbers, possibly facilitated by low pleiotropy (high *tau*) (Meisel 2011; Assis et al. 2012; Whittle et al. 2021; Fisher 1930). Crucially, however, such neutral (nondirectional) changes would not be expected to yield the striking patterns we found for gene-wide dN/dS per species in ovariole-related genes and ovariole numbers (across species; Tables 1 to 3), nor to give rise to the observed predictive relationships between dN/dS and ovariole numbers using PGLS (Table 4). Moreover, our explicit evidence of adaptive evolution across many ovariole-related genes, by gene-wide dN/dS values larger than 1, branch-site positive selection analysis and McDonald and Kreitman (1991) tests ($P < 0.05$, Tables 1 to 3, supplementary table S9, Supplementary Material online, Fig. 4, supplementary fig. S4, Supplementary Material online), is unlikely to be explained by neutral evolution alone. Thus, the present data suggest that neutral evolution has not been the only or main driving factor shaping amino acid changes in ovariole-related genes in the *melanogaster* group, which we propose instead are best explained by a history of adaptive evolution.

Another factor in addition to narrow expression breadth (a factor that affects individual genes) that could in theory lead to relaxed purifying selection on nonsynonymous mutations in ovariole genes is small population size, which may affect entire genomes (Kimura 1962; Strasburg et al. 2011; Gossmann et al. 2012). As an example, under this scenario, relaxed selection may be expected to be more common in the *D. sechellia* lineage (Fig. 2), in which the extant species has been suggested to have a smaller population size than other closely related *Drosophila* species such as *D. simulans* (Legrand et al. 2009). Thus, we do not exclude the possibility that certain gene-wide nonsynonymous changes (dN in dN/dS) in that species branch may have contributed to its altered ovariole numbers, under an assumption that some slightly deleterious mutations may behave as selectively neutral mutations (as effective population size (N_e) and selection coefficient (s) may yield, $N_e s < 1$) and be fixed by random genetic drift (Strasburg et al. 2011; Gossmann et al. 2012). However, as outlined above, the analyses showed affirmative branch-site positive selection tests here and the findings of gene-wide dN/dS values larger than 1 each control for neutral evolution (Zhang et al. 2005; Yang 2007) and showed that positive selection was common in the *D. sechellia* branch (Tables 1 and 2, supplementary table S7, Supplementary Material online, Fig. 4). Furthermore, the results revealed a heightened frequency of positive selection in genes upregulated in the TFs and SH cells in *D. sechellia* (Table 3, Fig. 4, supplementary table S7, Supplementary Material online), a pattern not explainable by neutral evolution (relaxed selection) due to population size. Collectively, the evidence suggests that relaxed purifying selection, while potentially accelerating divergence rates of some ovariole-related genes studied here (Duret and Mouchiroud 2000; Mank and Ellegren 2009; Meisel 2011; Whittle et al. 2021), may have its most significant role in the evolvability of ovariole-related genes (e.g. under high *tau*), enhancing the potential for adaptive evolution of protein sequences (Otto 2004; Larracuente et al. 2008; Mank and Ellegren 2009; Whittle et al. 2021) and in that manner potentially affecting interspecies ovariole number evolution.

Evolution of Multiple Developmental Processes via Rapid Divergence of Genes that Regulate Ovariole Number

Generating the right number of ovarioles for a given species relies on multiple developmental processes that begin during embryogenesis and are not completed until puparium formation. These include establishment of a specific number of somatic gonad precursor cells in the embryonic primordial gonad, proliferation at a specific rate and to a specific degree during larval stages, morphogenetic movements including intercalation and migration to establish TFs, and extracellular matrix deposition to separate ovarioles from each other within the gonad (King 1970). Any of these developmental processes could in principle be the target of evolutionary change in interspecies ovariole number divergence. Indeed, we previously showed that evolution of different developmental mechanisms underlies convergent evolution of similar ovariole numbers between or within species (Green and Extavour 2012). Accordingly, we would expect that the genes underlying these evolutionary changes might play roles in multiple different developmental processes, and this prediction is supported by our findings herein. The genes that we have identified here as not only rapidly evolving in the *melanogaster* subgroup (Tables 1 to 3), but also with molecular evolutionary rates that are highly predictive of

lineage-specific ovariole numbers (Table 4), have known functional roles in cell–cell signaling, cell proliferation, cell shape change, cell migration, and extracellular matrix composition and function (Table 3, supplementary table S8, Supplementary Material online; see gene descriptions in supplementary Text File S1, Supplementary Material online), including in but not limited to ovariole formation in *D. melanogaster*. Further, the distinct patterns of branch-site positive selection in different lineages suggest that ovariole number evolution involved modification of distinct developmental processes in different lineages. For example, the rapid evolution of *Zyx*, *vkg*, *col4a1*, *llp5,* and *CG3630* in the lineage leading to *D. sechellia* (Tables 1 to 3) suggests that alteration of the TF morphogenesis program was an important mechanism through which this species evolved its unusually low ovariole number (relative both to the other extant subgroup members and to its hypothesized last common ancestor; Green and Extavour 2012). In contrast, evolutionary changes in pathways such as JAK/STAT, Wnt, EGF, and Notch signaling pathways may have played a comparatively larger role in the evolution of more ovarioles in *D. simulans*, given the rapid evolution of *Su(var)2*, *CKIIbeta*, *vn*, *Gug*, and *E(spl)m2-BFM* along this branch (Table 1, supplementary table S3, Supplementary Material online).

Strengths and Limitations

The present study is strengthened by the fact that we assessed very closely related species within *melanogaster* subgroup, a clade that has been well defined phenotypically (including ovariole numbers), genetically, and phylogenetically (Hodin and Riddiford 2000; Starmer et al. 2003; Tamura et al. 2004; Markow et al. 2009; Obbard et al. 2012; Gramates et al. 2022; Fig. 2; and followed up with three Hawaiian species). We chose these closely related species to try to minimize the differences among species other than protein sequence divergence that may explain interspecies ovariole number evolution (Felsenstein 1985; Bromham et al. 1996; Whittle and Johnston 2003; Thomas et al. 2010; Symonds and Blomberg 2014). Moreover, the closely related species allowed us to study a high number of interspecies orthologs (as we had to contend with fewer gene gains/losses and fewer genes with orthologs too divergent to identify, than would have been the case with more divergent systems; Stanley and Kulathinal 2016), and ensured that dN and dS were each unsaturated (see Materials and Methods), such that dN/dS (supplementary fig. S1, Supplementary Material online) would be more likely to accurately reflect selection pressures (Yang and Nielsen 2002; Yang 2007). Further, by using a targeted approach whereby ovariole-related genes were prescreened in one species (*D. melanogaster*) using functional genetic and single-cell-resolution expression methods (Kumar et al. 2020; Slaidina, Gupta et al. 2020; Tarikere et al. 2022), it strengthens the inference that the rapidly evolving ovariole-related genes (Tables to 3) are associated with evolved changes in ovariole numbers. This targeted approach may allow a more cost-efficient, time-efficient, and effective means to identify genes putatively involved in phenotype evolution than methods that broadly scan entire genomes across many highly divergent species, and provide a set of high-confidence gene sets for follow-up analyses.

In terms of limitations, we note that the 42 ovariole-related genes that were identified as strong candidates for shaping ovariole number divergence are limited to the specific species of the *melanogaster* subgroup studied here (Fig. 2; with notably, some inferences ascertained for the Hawaiian clade; supplementary fig. S6, Supplementary Material online). It will be valuable in the future to assess the generalizability of these patterns by investigating other closely related species' clades of *Drosophila*, or of other insects, and/or using other approaches including phylogenetically independent contrasts (Whittle and Johnston 2003; Whittle and Extavour 2016; Dunn et al. 2018) as more data become available on the genetic regulation of ovariole number, species genomes, and ovariole numbers per species. This may provide insights into whether the same or different genes underlie ovariole number evolution in diverse taxa.

Another caveat is that we studied only those genes with orthologs identifiable in all five *melanogaster* subgroup species, and thus excluded genes with gains or losses and with extreme sequence divergence (Tautz and Domazet-Loso 2011; Tautz et al. 2013), that may also have substantially contributed toward interspecies changes in ovariole numbers. Further, it is worth noting that while the *melanogaster* subgroup species studied here were all very closely related to *D. melanogaster*, which was the species used for experimental and transcriptional analysis to identify ovariole-involved genes (RNAi and sc-RNA-seq data), we do not exclude the possibility that there may be differences in the expression of these genes among species. In fact, for the 42 ovariole-related genes of interest, the observed rapid functional protein sequence changes (dN/dS) and their association with interspecies shifts in ovariole numbers suggest that there has been modification of their function within the ovarioles among species (and was a goal of this study to identify genes matching these criteria). In turn, a gene may require fewer or more cellular transcripts under its modified function, and this may lead to the evolution of expression levels among species. Protein sequence divergence among species has been correlated with gene expression changes in various systems (Khaitovich et al. 2005; Warnefors and Kaessmann 2013; Whittle and Johannesson 2013), suggesting that for some ovariole genes it is possible that both the protein sequence and transcript levels may have shifted at the interspecies level, in conjunction with ovariole numbers. Together, our data suggest a group of ovariole genes for such follow-up research, including assessments of gene expression, gene function, and transcriptome changes with respect to ovariole numbers.

Future Directions

The present study reveals a set of ovariole-involved genes, with established roles in ovariole numbers and functions, whose protein sequence divergence suggests a substantive link to ovariole number divergence in the *D. melanogaster* subgroup, based on a multilayered analysis of branch dN/dS, branch-site analyses, *tau*, and PGLS. For many genes, the branch dN/dS value was predictive of ovariole numbers among species (Table 4), consistent with an interdependent relationship. Further, our analyses of ovariole-involved genes in the Hawaiian *Drosophila* clade suggest that protein divergence of ovariole-related genes may shape ovariole number changes broadly across disparate clades of the *Drosophila* genus (supplementary table S10 and fig. S6, Supplementary Material online). The molecular evolutionary approach used herein may provide valuable opportunities for the discovery of genes and evolutionary processes involved in interspecies phenotype divergence, particularly important for reproductive and fitness-related traits (Dorus et al. 2004; Nadeau et al. 2007; Ramm et al. 2008; Wlasiuk and Nachman 2010; Luke et al. 2014; Corso et al. 2016; Chebbo et al. 2021), which remains a central challenge in evolutionary developmental biology (Hoekstra and Coyne 2007; Cutter and Bundus 2020).

We suggest that future examinations of the genetic basis of interspecies divergence in ovariole number and other related reproductive traits will be most fruitfully pursued along one or more of the following major directions: First, assessments of protein sequence changes in ovariole-related genes identified here at the population level using genome-wide association studies and mutational frequency spectra (Akashi 1997; Whittle et al. 2012; Lobell et al. 2017), combined with McDonald–Kreitman tests (McDonald and Kreitman 1991; Murga-Moreno et al. 2019), for multiple *Drosophila* species, will help discern evolutionary dynamics of these genes at the microevolutionary scale. Second, studies of gene expression divergence and the functional roles (RNAi) of the 42 ovariole-related genes that were identified as strong candidates to shape ovariole number divergence should be conducted in the *melanogaster* subgroup species outside of *D. melanogaster* (*D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*). Such analyses will provide insights into interrelationships between protein sequence evolution, gene expression changes, gene function modifications, and shifts in ovariole numbers (Tables 1 to 3). Third, further studies should consider the roles of gene losses and gains in *Drosophila* lineages (Coyne and Hoekstra 2007; Tautz and Domazet-Loso 2011; Tautz et al. 2013), as well as by genes that have diverged too rapidly to allow identification of orthologs (supplementary fig. S6, Supplementary Material online) (Tautz and Domazet-Loso 2011; Tautz et al. 2013). Fourth, studies on the mating behaviors and sexual selection pressures, including male-mate choice, female competition, and sexual antagonism, in species of the *melanogaster* subgroup (Bonduriansky 2001; Sirot et al. 2014; Bath et al. 2018; Veltsos et al. 2022), will be valuable to revealing their possible links to ovariole numbers. Finally, further research should include studies in the Hawaiian *Drosophila*, given our results suggest protein divergence of numerous ovariole-related genes may contribute to ovariole number changes in the three-species Hawaiian clade studied herein (supplementary table S10 and supplementary figs. S5 and S6, Supplementary Material online). The Hawaiian group is known for its wide phenotypic diversity in sexual characteristics, ranging from behaviors to ovariole numbers (Carson 1997; Singh and Singh 2014; Sarikaya et al. 2019). Studies on the relationships between protein sequence changes and ovariole numbers in Hawaiian *Drosophila* will be facilitated by increased collection of whole genomes and transcriptomic data for the larval ovaries, including TFs and SH cells, and potentially by the use of expanding tools aimed to correlate gene and phenotype evolution (Kowalczyk et al. 2019). Such research will help further decipher the genetic factors shaping the rapid evolution of ovariole numbers in the *Drosophila* genus, and thus in insects more broadly.

Materials and Methods

Identification of Rapidly Evolving Ovariole-Related Genes for Follow-Up Analyses

For the SIGNALC gene set, which was based on *D. melanogaster* RNAi data (Kumar et al. 2020), we screened the 67 genes that directly affected ovariole numbers, named *hpo[RNAi]* Ovariole Number, 59 and 49 genes that affected egg laying, named *hpo[RNAi]* Egg Laying and Egg Laying *[wt]*, and the 17 connector genes. For these four SIGNALC genes sets, we identified any genes with M0 dN/dS $\geq$1.5 higher than the genome-wide median. The cut-off was marginally lower than the BULKSG and SINGLEC because of the innate conserved nature of these signaling pathway genes, which are largely at least as old as animal divergence, in excess of 600 million years (Srivastava et al. 2010; Kumar et al. 2020). For the BULKSG dataset (Tarikere et al. 2022), we screened for any differentially expressed genes that had M0 dN/dS $\geq$ 0.20 in the *melanogaster* subgroup for further study. This represents a value $\geq$2.2 higher than the genome-wide median. With respect to the SINGLEC dataset (Slaidina, Gupta et al. 2020), for the genes with differential expression in one cell type relative to the others ($P < 0.05$), we identified those with M0 dN/dS $\geq$ 0.20, similar to the BULKSG dataset. The M0 dN/dS values for the five species under study in the *melanogaster* subgroup were from FlyDivas (Stanley and Kulathinal 2016) that matched our own M0 dN/dS calculations in PAML (Yang 2007) (additional details on screening are available in supplementary Text File S1, Supplementary Material online).

Follow-Up Assessments: dN/dS Per Species Terminal Branch, Branch-Site Positive Selection, and *tau*

Determining dN/dS for Each Species Terminal Branch

We calculated the M1 free ratios dN/dS per species terminal branch using codeml package in PAML (Yang 2007), which allows a separate dN/dS value for each branch, using as input publicly available high-confidence genome-wide five-species sequence alignments from FlyDivas, which has data for various species groups of *Drosophila* (Stanley and Kulathinal 2016). Codeml is based on maximum likelihood (ML) in deriving estimates of dN/dS values, and default parameters were used in the assessments (Yang 2007). Using the dN/dS for each of the five terminal species branches, we assessed associations with respect to species transitions in ovariole numbers (terminal species branch analysis), an approach that has proven effective for determining relationships between dN/dS values and phenotypes of interest (Dorus et al. 2004; Nadeau et al. 2007; Wlasiuk and Nachman 2010).

We assessed the distributions of dN/dS for all studied genes per species branch (supplementary fig. S1, Supplementary Material online). To affirm the suitability of the obtained data to determine dN/dS in each individual species terminal branch, we examined the magnitude of dN and dS values. The vast majority of genes had dN and dS <1.5 per species terminal branch and thus were unsaturated: 99.95% and 99.5% of genes in *D. simulans*, respectively, had values below this threshold, and we found even higher percentages (up to 100%) for the four other species. Only gene branches that had dN or dS >0.001 were included for further assessment to ensure sufficient divergence for study (Cusack and Wolfe 2007; Whittle et al. 2021). The minority of cases of a branch where dN was >0.001 and dS was at or near zero were denoted simply as "dN/dS > 1" (e.g. 0.2% of all 9,232 genes studied in *D. melanogaster*), rather than infinity (see also other approaches to cases of dS near 0 and dN > 0 (Wlasiuk and Nachman 2010), and were interpreted conservatively.

Branch-Site Positive Selection Analysis

Branch-site codon analysis was used to assess positive selection at specific codon sites for each species terminal branch of the *melanogaster* subgroup (Fig. 2) as described in the PAML manual (Yang and Nielsen 2002; Zhang et al. 2005; Yang 2007). For all aligned genes from the *melanogaster* subgroup ($N = 9{,}237$ alignments; note 9,232 had M0 values for study) (Stanley and Kulathinal 2016), including for the identified rapidly evolving ovariole-related genes, one of the five *Drosophila* species was assigned as the foreground branch in its own individual branch-site analysis. Thus, a separate branch-site analysis was conducted for all studied genes for *D. simulans*, *D. sechellia*, *D. melanogaster*, *D. yakuba*, and *D. erecta*. For each gene, the ML values were compared between a model with and without branch-site positive selection (codeml model = 2, NSsites = 2, with fix_omega = 1 vs. 0, and *P* value of χ^2 for 2XΔlnL). *P* values <0.05 for 2XΔlnL for any gene were interpreted as evidence of positive selection at one or more codon sites in that species branch. We studied the presence or absence of branch-site positive selection within each gene, suggested by Zhang et al. (2005), without including the post hoc option for BEB probability analysis per codon site that has low power (Zhang et al. 2005). The frequency of genes with branch-site positive selection in the ovariole-related gene sets under study was compared to the genome-wide frequency per species branch. Multiple test corrections were not applied as this was deemed overly conservative for our purposes of identification of ovariole-related genes with signals of positive selection, and these results were combined with other multiple layers of analyses (branch dN/dS, *tau*, and PGLS). The input tree for branch and branch-site analysis was an unrooted Newick phylogeny (unrooted version of Fig. 2) as required by PAML (Yang 2007).

Expression Specificity Quantification Using tau

We used the index *tau* to measure expression specificity of the genes under study here (Yanai et al. 2005). For this, we accessed expression data from 59 tissue types and developmental stages from *D. melanogaster* (30 developmental stages and 29 tissues, supplementary table S1, Supplementary Material online). The data include gene expression levels in reads per kilobase million (RPKM) across development for embryos (12 stages), larvae (6 stages), pupae (6 stages), and adults (3 stages of males/females) and for major tissue types of the adult males and females (including heads, gonads, and central nervous system). The expression data were from modEncode and included the RNA-seq datasets generated by Graveley et al. (2011) (available at: https://flybase.org/commentaries/2013_05/rna-seq_bulk.html; downloaded March 2022; see also supplementary Text File S1, Supplementary Material online) which comprise among the widest scope of expression data available in insects (Li et al. 2014). The *tau* value per gene was calculated as follows:

$$tau(\tau) = \frac{\sum_{i=1}^{n}(1-\hat{x}_i)}{n-1} \; ; \; \hat{x}_i = x_i / max(x_i)$$

where n is the number of tissues/stages studied, i is the tissue/stage, x_i is the expression level of gene in tissue/stage i, and max (x_i) is the expression level in the tissue/stage type with maximum expression (Yanai et al. 2005).

Elevated values in one gene relative to another indicate greater expression specificity, such that most transcripts originate from few tissues/stages (see supplementary fig. S2

and supplementary Text File S1, Supplementary Material online for an overview of the genome-wide *tau* values herein). Genes with *tau* values above 0.90 were considered highly specific in expression.

PGLS Analysis

PGLS was assessed for ovariole number (dependent parameter) with respect to branch dN/dS (independent parameter) using the five terminal species branches of the *melanogaster* subgroup (Fig. 2). PGLS was conducted using the comparative analysis of phylogenetics and evolution (Caper) package available in R (R-Core-Team 2022) (https://cran.r-project.org/web/packages/caper/index.html). The covariance matrix of species relationships was obtained under the assumption of Brownian motion using the vcv function in caper. Under a five-species tree, any genes showing $P < 0.05$ suggest a strong relationship between ovariole number and dN/dS, sufficient to be detected under this sample size. In turn, $P > 0.05$ does not necessarily preclude a relationship, which may be inferred from our combined analysis of dN/dS, positive selection analysis, and *tau*. The phylogenetic tree used for the covariance matrix in PGLS is shown in Fig. 2.

McDonald–Kreitman Tests

We conducted McDonald and Kreitman (1991) tests for genes of interest using the integrative McDonald and Kreitman test (iMKT) database (Murga-Moreno et al. 2019). For these tests, we examined the Raleigh NC and Zambia populations, and the interspecies divergence was conducted using *D. melanogaster–D. simulans* contrasts (Murga-Moreno et al. 2019). Thus, this analysis tests positive selection since divergence of the *D. melanogaster–D. simulans* branches only.

Drosophila *Phylogeny*

To obtain the phylogeny for the five-species *melanogaster* subgroup in Fig. 2, we used aligned sequence data from DrosoPhyla (Finet et al. 2021) that contains a prescreened dataset of 17 genes across 704 species of *Drosophilidae* (which were screened for quality, sufficient divergence, and phylogenetic informativeness). We extracted the concatenated aligned sequences for *D. simulans*, *D. sechellia*, *D. melanogaster*, *D. yakuba*, and *D. erecta*, included *D. ananassae* as an outgroup as a reference (for the phylogeny construction), and removed all gaps and any sites with unknown nucleotides, yielding a total of 9,235 nucleotide sites. Using MEGA11 (Tamura et al. 2021), we generated a ML phylogenetic tree, including the tree lengths, based on the default parameters. We also obtained a tree using the neighbor-joining method, with nearly identical results. The relative relationships of the species in the obtained trees matched those previously observed for these five species (Obbard et al. 2012; Finet et al. 2021).

Hierarchical Clustering of Expression in the SINGLEC Dataset

The relationships in gene expression across the nine different cell types of the *D. melanogaster* LL3 ovary (Fig. 1A) from the SINGLEC dataset (Slaidina, Gupta et al. 2020) were assessed using hierarchical clustering under the average linkage method applied to the average standardized expression values per gene for all genes with nonzero expression (determined in Suerat v2, see Slaidina et al. 2020). The analysis was conducted in the Morpheus program (https://software.broadinstitute.org/morpheus).

Gene Ontology

To study inferred gene functions and the clustering of genes by inferred function, we used the program DAVID (Huang et al. 2009), which provides inferred gene function data for *D. melanogaster* using the FlyBase gene identifiers (Gramates et al. 2022).

Supplementary Analyses of a Three-Species Hawaiian Clade

We followed up on our main assessments of the *melanogaster* subgroup, with a supplementary evaluation of ovariole numbers and ovariole-related gene dN/dS in a three-species clade from the distantly related Hawaiian *Drosophila* that included *D. sproati*, *D. murphyi*, and *D. grimshawi*. The methods applied for CDS extraction, ortholog identification, gene alignments, and dN/dS analyses for that assessment are described in supplementary Text File S1, Supplementary Material online.

Supplementary Material

Supplementary material is available at *Genome Biology and Evolution* online.

Acknowledgments

The authors thank members of the Extavour Lab for valuable discussions. The experimental and transcriptome data generated by cited research of Dr. Tarun Kumar, Dr. Leo Blondel, Dr. Shreeharsha Tarikere, and Dr. Guillem Ylla that allowed prescreening of genes for ovariole functions are appreciated, as well as by the authors of the sc-RNA-seq datasets cited in the Materials and Methods. The authors also appreciate valuable comments by the anonymous reviewers that helped improve the manuscript.

Author Contributions

C.A.W. and C.G.E. conceived the study, wrote the manuscript, and conducted analysis.

Data Availability

All data used in the present study are publicly available as described in Materials and Methods and supplementary Text File S1, Supplementary Material online.

Literature Cited

Akashi H. Codon bias evolution in *Drosophila*. Population genetics of mutation-selection drift. Gene. 1997:205(1-2):269–278. https://doi.org/10.1016/s0378-1119(97)00400-9.

Arnqvist G, Rock L. Sexual conflict and arms races between the sexes: a morphological adaptation for control of mating in a female insect. Proc Royal Soc Sec B: Biol. 1995:261(1360):123–127. https://doi.org/10.1098/rspb.1995.0126.

Assis R, Zhou Q, Bachtrog D. Sex-biased transcriptome evolution in *Drosophila*. Genome Biol Evol. 2012:4(11):1189–1200. https://doi.org/10.1093/gbe/evs093.

Bath E, Morimoto J, Wigby S. The developmental environment modulates mating-induced aggression and fighting success in adult female *Drosophila*. Funct Ecol. 2018:32(11):2542–2552. https://doi.org/10.1111/1365-2435.13214.

Bergland AO, Genissel A, Nuzhdin SV, Tatar M. Quantitative trait loci affecting phenotypic plasticity and the allometric relationship of ovariole number and thorax length in *Drosophila melanogaster*. Genetics. 2008:180(1):567–582. https://doi.org/10.1534/genetics.108.088906.

Bielawski JP, Yang Z. Likelihood methods for detecting adaptive evolution. In: Nielsen R, editors. Statistical methods in molecular evolution. New York: Springer Verlag; 2005. p. 103–124.

Bonduriansky R. The evolution of male mate choice in insects: a synthesis of ideas and evidence. Biol Rev Camb Philos Soc. 2001:76(3):305–339. https://doi.org/10.1017/s1464793101005693.

Borges R, Fonseca J, Gomes C, Johnson WE, O'Brien SJ, Zhang G, Gilbert MTP, Jarvis ED, Antunes A. Avian binocularity and adaptation to nocturnal environments: genomic insights from a highly derived visual phenotype. Genome Biol Evol. 2019:11(8):2244–2255. https://doi.org/10.1093/gbe/evz111.

Bromham L, Rambaut A, Harvey PH. Determinants of rate variation in mammalian DNA sequence evolution. J Mol Evol. 1996:43(6):610–621. https://doi.org/10.1007/bf02202109.

Bubnell JE, Ulbing CKS, Fernandez Begne P, Aquadro CF. Functional divergence of the bag-of-marbles gene in the *Drosophila* melanogaster species group. Mol Biol Evol. 2022:39(7):msac137. https://doi.org/10.1093/molbev/msac137.

Buschiazzo E, Ritland C, Bohlmann J, Ritland K. Slow but not low: genomic comparisons reveal slower evolutionary rate and higher dN/dS in conifers compared to angiosperms. BMC Evol Biol. 2012:12(1):8. https://doi.org/10.1186/1471-2148-12-8.

Byrne PG, Rice WR. Evidence for adaptive male mate choice in the fruit fly *Drosophila melanogaster*. Proc Biol Sci. 2006:273(1589):917–922. https://doi.org/10.1098/rspb.2005.3372.

Carson HL. The Wilhelmine E. Key 1996 invitational lecture. Sexual selection: a driver of genetic change in Hawaiian *Drosophila*. J Hered. 1997:88(5):343–352. https://doi.org/10.1093/oxfordjournals.jhered.a023115.

Castillo-Davis CI, Bedford TB, Hartl DL. Accelerated rates of intron gain/loss and protein evolution in duplicate genes in human and mouse malaria parasites. Mol Biol Evol. 2004:21(7):1422–1427. https://doi.org/10.1093/molbev/msh143.

Chapman T, Herndon LA, Heifetz Y, Partridge L, Wolfner MF. The Acp26Aa seminal fluid protein is a modulator of early egg hatchability in *Drosophila melanogaster*. Proc Biol Sci. 2001:268(1477):1647–1654. https://doi.org/10.1098/rspb.2001.1684.

Chebbo S, Josway S, Belote JM, Manier MK. A putative novel role for Eip74EF in male reproduction in promoting sperm elongation at the cost of male fecundity. J Exp Zool B Mol Dev Evol. 2021:336(8):620–628. https://doi.org/10.1002/jez.b.22986.

Church SH, de Medeiros BAS, Donoughe S, Marquez Reyes NL, Extavour CG. Repeated loss of variation in insect ovary morphology highlights the role of development in life-history evolution. Proc Biol Sci. 2021:288(1950):20210150. https://doi.org/10.1098/rspb.2021.0150.

Civetta A, Clark AG. Correlated effects of sperm competition and postmating female mortality. Proc Nat Acad Sci U S A. 2000:97(24):13162–13165. https://doi.org/10.1073/pnas.230305397.

Civetta A, Singh RS. Sex-related genes, directional sexual selection, and speciation. Mol Biol Evol. 1998:15(7):901–909. https://doi.org/10.1093/oxfordjournals.molbev.a025994.

Clark NL, Gasper J, Sekino M, Springer SA, Aquadro CF, Swanson WJ. Coevolution of interacting fertilization proteins. PLoS Genet. 2009:5(7):e1000570. https://doi.org/10.1371/journal.pgen.1000570.

Clark IB, Jarman AP, Finnegan DJ. Live imaging of *Drosophila* gonad formation reveals roles for Six4 in regulating germline and somatic cell migration. BMC Dev Biol. 2007:7(1):52. https://doi.org/10.1186/1471-213X-7-52.

Corso J, Mundy NI, Fagundes NJ, de Freitas TR. Evolution of dark colour in toucans (Ramphastidae): a case of molecular adaptation? J Evol Biol. 2016:29(12):2530–2538. https://doi.org/10.1111/jeb.12982.

Coyne JA, Charlesworth B. Genetics of a pheromonal difference affecting sexual isolation between *Drosophila mauritiana* and *D. sechellia*. Genetics. 1997:145(4):1015–1030. https://doi.org/10.1093/genetics/145.4.1015.

Coyne JA, Hoekstra HE. Evolution of protein expression: new genes for a new diet. Curr Biol. 2007:17(23):R1014–R1016. https://doi.org/10.1016/j.cub.2007.10.009.

Coyne JA, Rux J, David JR. Genetics of morphological differences and hybrid sterility between *Drosophila sechellia* and its relatives. Genet Res. 1991:57(2):113–122. https://doi.org/10.1017/s0016672300029177.

Cui Q, Purisima EO, Wang E. Protein evolution on a human signaling network. BMC Sys Biol. 2009:3(1):21. https://doi.org/10.1186/1752-0509-3-21.

Cusack BP, Wolfe KH. Not born equal: increased rate asymmetry in relocated and retrotransposed rodent gene duplicates. Mol Biol Evol. 2007:24(3):679–686. https://doi.org/10.1093/molbev/msl199.

Cutter AD. Divergence times in *Caenorhabditis* and *Drosophila* inferred from direct estimates of the neutral mutation rate. Mol Biol Evol. 2008:25(4):778–786. https://doi.org/10.1093/molbev/msn024.

Cutter AD, Bundus JD. Speciation and the developmental alarm clock. Elife. 2020:9:e56276. https://doi.org/10.7554/eLife.56276.

Dansereau DA, Lasko P. The development of germline stem cells in *Drosophila*. Methods Mol Biol. 2008:450:3–26. https://doi.org/10.1007/978-1-60327-214-8_1.

Dean R, Mank JE. Tissue specificity and sex-specific regulatory variation permit the evolution of sex-biased gene expression. Am Nat. 2016:188(3):E74–E84. https://doi.org/10.1086/687526.

Dorus S, Evans PD, Wyckoff GJ, Choi SS, Lahn BT. Rate of molecular evolution of the seminal protein gene SEMG2 correlates with levels of female promiscuity. Nat Genet. 2004:36(12):1326–1329. https://doi.org/10.1038/ng1471.

Dunn CW, Zapata F, Munro C, Siebert S, Hejnol A. Pairwise comparisons across species are problematic when analyzing functional genomic data. Proc Nat Acad Sci U S A. 2018:115(3): E409–E417. https://doi.org/10.1073/pnas.1707515115.
Duret L, Mouchiroud D. Determinants of substitution rates in mammalian genes: expression pattern affects selection intensity but not mutation rate. Mol Biol Evol. 2000:17(1):68–74. https://doi.org/10.1093/oxfordjournals.molbev.a026239.
Eliazer S, Buszczak M. Finding a niche: studies from the *Drosophila* ovary. Stem Cell Res Therapy. 2011:2(6):45. https://doi.org/10.1186/scrt86.
Extavour CG, Akam ME. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development. 2003:130(24):5869–5884. https://doi.org/10.1242/dev.00804.
Felsenstein J. Phylogenies and the comparative method. Am Nat. 1985:125(1):1–15. https://doi.org/10.1086/284325.
Finet C, Kassner VA, Carvalho AB, Chung H, Day JP, Day S, Delaney EK, De Ré FC, Dufour HD, Dupim E, et al. DrosoPhyla: resources for drosophilid phylogeny and systematics. Genome Biol Evol. 2021:13(8):evab179. https://doi.org/10.1093/gbe/evab179.
Fisher RA. The genetical theory of natural selection. Oxford: Clarendon Press; 1930.
Friberg U, Arnqvist G. Fitness effects of female mate choice: preferred males are detrimental for *Drosophila melanogaster* females. J Evol Biol. 2003:16(5):797–811. https://doi.org/10.1046/j.1420-9101.2003.00597.x.
Gilboa L, Lehmann R. Soma-germline interactions coordinate homeostasis and growth in the *Drosophila* gonad. Nature. 2006:443(7107):97–100. https://doi.org/10.1038/nature05068.
Godt D, Laski FA. Mechanisms of cell rearrangement and cell recruitment in *Drosophila* ovary morphogenesis and the requirement of bric a brac. Development. 1995:121(1):173–187. https://doi.org/10.1242/dev.121.1.173.
Goldman-Huertas B, Mitchell RF, Lapoint RT, Faucher CP, Hildebrand JG, Whiteman NK. Evolution of herbivory in Drosophilidae linked to loss of behaviors, antennal responses, odorant receptors, and ancestral diet. Proc Nat Acad Sci U S A. 2015:112(10): 3026–3031. https://doi.org/10.1073/pnas.1424656112.
Gossmann TI, Keightley PD, Eyre-Walker A. The effect of variation in the effective population size on the rate of adaptive molecular evolution in eukaryotes. Genome Biol Evol. 2012:4(5):658–667. https://doi.org/10.1093/gbe/evs027.
Gramates LS, Agapite J, Attrill H, Calvi BR, Crosby MA, Santos GD, Goodman JL, Goutte-Gattat D, Jenkins VK, Kaufman T, et al. FlyBase: a guided tour of highlighted features. Genetics. 2022: 220(4):iyac035. https://doi.org/10.1093/genetics/iyac035.
Graveley BR, Brooks AN, Carlson JW, Duff MO, Landolin JM, Yang L, Artieri CG, van Baren MJ, Boley N, Booth BW, et al. The developmental transcriptome of *Drosophila melanogaster*. Nature. 2011:471(7339):473–479. https://doi.org/10.1038/nature09715.
Green DA, 2nd, Extavour CG. Convergent evolution of a reproductive trait through distinct developmental mechanisms in *Drosophila*. Dev Biol. 2012:372(1):120–130. https://doi.org/10.1016/j.ydbio.2012.09.014.
Haerty W, Jagadeeshan S, Kulathinal RJ, Wong A, Ram KR, Sirot LK, Levesque L, Artieri CG, Wolfner MF, Civetta A, et al. Evolution in the fast lane: rapidly evolving sex-related genes in *Drosophila*. Genetics. 2007:177(3):1321–1335. https://doi.org/10.1534/genetics.107.078865.
Ho WW, Smith SD. Molecular evolution of anthocyanin pigmentation genes following losses of flower color. BMC Evol Biol. 2016:16(1): 98. https://doi.org/10.1186/s12862-016-0675-3.
Hodin J, Riddiford LM. Different mechanisms underlie phenotypic plasticity and interspecific variation for a reproductive character in drosophilids (Insecta: Diptera). Evolution. 2000:54(5): 1638–1653. https://doi.org/10.1111/j.0014-3820.2000.tb00708.x.
Hoekstra HE, Coyne JA. The locus of evolution: evo devo and the genetics of adaptation. Evolution. 2007:61(5):995–1016. https://doi.org/10.1111/j.1558-5646.2007.00105.x.
Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Prot. 2009:4(1):44–57. https://doi.org/10.1038/nprot.2008.211.
Innocenti P, Morrow EH. The sexually antagonistic genes of *Drosophila melanogaster*. PLoS Biol. 2010:8(3):e1000335. https://doi.org/10.1371/journal.pbio.1000335.
Jagadeeshan S, Singh RS. Rapidly evolving genes of *Drosophila*: differing levels of selective pressure in testis, ovary, and head tissues between sibling species. Mol Biol Evol. 2005:22(9):1793–1801. https://doi.org/10.1093/molbev/msi175.
Jagadeeshan S, Singh RS. Rapid evolution of outer egg membrane proteins in the *Drosophila melanogaster* subgroup: a case of ecologically driven evolution of female reproductive traits. Mol Biol Evol. 2007:24(4):929–938. https://doi.org/10.1093/molbev/msm009.
Kambysellis MP, Heed WB. Studies of oogenesis in natural populations of drosophilidae. I. Relation of ovarian development and ecological habitats of the Hawaiian Species. Am Soc Nat. 1971:105(941): 31–49. https://doi.org/10.1086/282700.
Kambysellis MP, Ho K-F, Craddock EM, Piano F, Parisi M, Cohen J. Pattern of ecological shifts in the diversification of Hawaiian *Drosophila* inferred from a molecular phylogeny. Curr Biol. 1995:5(10): 1129–1139. https://doi.org/10.1016/s0960-9822(95)00229-6.
Kaneshiro KY, Boake CR. Sexual selection and speciation: issues raised by Hawaiian *Drosophila*. Trends Ecol Evol. 1987:2(7):207–212. https://doi.org/10.1016/0169-5347(87)90022-X.
Kang L, Settlage R, McMahon W, Michalak K, Tae H, Garner HR, Stacy EA, Price DK, Michalak P. Genomic signatures of speciation in sympatric and allopatric Hawaiian picture-winged *Drosophila*. Genome Biol Evol. 2016:8(5):1482–1488. https://doi.org/10.1093/gbe/evw095.
Khaitovich P, Hellmann I, Enard W, Nowick K, Leinweber M, Franz H, Weiss G, Lachmann M, Pääbo S. Parallel patterns of evolution in the genomes and transcriptomes of humans and chimpanzees. Science. 2005:309(5742):1850–1854. https://doi.org/10.1126/science.1108296.
Kim PM, Korbel JO, Gerstein MB. Positive selection at the protein network periphery: evaluation in terms of structural constraints and cellular context. Proc Nat Acad Sci U S A. 2007:104(51):20274–-20279. https://doi.org/10.1073/pnas.0710183104.
Kim BY, Wang JR, Miller DE, Barmina O, Delaney E, Thompson A, Comeault AA, Peede D, D'Agostino ERR, Pelaez J, et al. Highly contiguous assemblies of 101 drosophilid genomes. Elife. 2021:10: e66405. https://doi.org/10.7554/eLife.66405.
Kimura M. On the probability of fixation of mutant genes in a population. Genetics. 1962:47(6):713–719. https://doi.org/10.1093/genetics/47.6.713.
Kimura M. The neutral theory of molecular evolution. Cambridge: Cambridge University Press; 1983.
Kimura M. The neutral theory of molecular evolution and the world view of the neutralists. Genome. 1989:31(1):24–31. https://doi.org/10.1139/g89-009.
King RC. Ovarian development in Drosophila melanogaster. New York: Academic Press; 1970.
King RC, Aggarwal SK, Aggarwal U. The development of the female *Drosophila* reproductive system. J Morphol. 1968:124(2): 143–166. https://doi.org/10.1002/jmor.1051240203.
Kiss AA, Somlyai-Popovics N, Kiss M, Boldogkői Z, Csiszár K, Mink M. Type IV collagen is essential for proper function of

integrin-mediated adhesion in Drosophila muscle fibers. Int J Mol Sci. 2019:20(20):5124. https://doi.org/10.3390/ijms20205124.
Kong HG, Kim HH, Chung J-H, Jun J, Lee S, Kim H-M, Jeon S, Park SG, Bhak J, Ryu C-M. The *Galleria mellonella* hologenome supports microbiota-independent metabolism of long-chain hydrocarbon beeswax. Cell Rep. 2019:26(9):2451–2464.e2455. https://doi.org/10.1016/j.celrep.2019.02.018.
Kowalczyk A, Meyer WK, Partha R, Mao W, Clark NL, Chikina M. RERconverge: an R package for associating evolutionary rates with convergent traits. Bioinformatics. 2019:35(22):4815–4817. https://doi.org/10.1093/bioinformatics/btz468.
Kumar T, Blondel L, Extavour CG. Topology-driven protein-protein interaction network analysis detects genetic sub-networks regulating reproductive capacity. Elife. 2020:9:e54082. https://doi.org/10.7554/eLife.54082.
LaBella AL, Opulente DA, Steenwyk JL, Hittinger CT, Rokas A. Signatures of optimal codon usage in metabolic genes inform budding yeast ecology. PLoS Biol. 2021:19(4):e3001185. https://doi.org/10.1371/journal.pbio.3001185.
Lande R. Models of speciation by sexual selection on polygenic traits. Proc Nat Acad Sci U S A. 1981:78(6):3721–3725. https://doi.org/10.1073/pnas.78.6.3721.
Larracuente AM, Sackton TB, Greenberg AJ, Wong A, Singh ND, Sturgill D, Zhang Y, Oliver B, Clark AG. Evolution of protein-coding genes in *Drosophila*. Trend Genet. 2008:24(3):114–123. https://doi.org/10.1016/j.tig.2007.12.001.
Lebo DPV, McCall K. Murder on the ovarian express: a tale of non-autonomous cell death in the *Drosophila* ovary. Cells. 2021:10(6):1454. https://doi.org/10.3390/cells10061454.
Legrand D, Tenaillon MI, Matyot P, Gerlach J, Lachaise D, Cariou M-L. Species-wide genetic variation and demographic history of *Drosophila sechellia*, a species lacking population structure. Genetics. 2009:182(4):1197–1206. https://doi.org/10.1534/genetics.108.092080.
LeVasseur-Viens H, Polak M, Moehring AJ. No evidence for external genital morphology affecting cryptic female choice and reproductive isolation in *Drosophila*. Evolution. 2015:69(7):1797–1807. https://doi.org/10.1111/evo.12685.
Li MA, Alls JD, Avancini RM, Koo K, Godt D. The large maf factor traffic jam controls gonad morphogenesis in *Drosophila*. Nat Cell Biol. 2003:5(11):994–1000. https://doi.org/10.1038/ncb1058.
Li JJ, Huang H, Bickel PJ, Brenner SE. Comparison of *D. melanogaster* and *C. elegans* developmental stages, tissues, and cells by modENCODE RNA-seq data. Genome Res. 2014:24(7):1086–1101. https://doi.org/10.1101/gr.170100.113.
Lobell AS, Kaspari RR, Serrano Negron YL, Harbison ST. The genetic architecture of ovariole number in *Drosophila melanogaster*: genes with Major, quantitative, and pleiotropic effects. G3 (Bethesda). 2017:7(7):2391–2403. https://doi.org/10.1534/g3.117.042390.
Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014:15(12):550. https://doi.org/10.1186/s13059-014-0550-8.
Luke L, Vicens A, Tourmente M, Roldan ER. Evolution of protamine genes and changes in sperm head phenotype in rodents. Biol Rep. 2014:90(3):67. https://doi.org/10.1095/biolreprod.113.115956.
Lupold S, Manier MK, Puniamoorthy N, Schoff C, Starmer WT, Luepold SHB, Belote JM, Pitnick S. How sexual selection can drive the evolution of costly sperm ornamentation. Nature. 2016:533(7604):535–538. https://doi.org/10.1038/nature18005.
Macagno AL, Beckers OM, Moczek AP. Differentiation of ovarian development and the evolution of fecundity in rapidly diverging exotic beetle populations. J Exp Zool. Part A: Ecol Genet Physiol. 2015:323(9):679–688. https://doi.org/10.1002/jez.1959.
Mank JE, Ellegren H. Are sex-biased genes more dispensable? Biol Lett. 2009:5(3):409–412. https://doi.org/10.1098/rsbl.2008.0732.
Mank JE, Hultin-Rosenberg L, Zwahlen M, Ellegren H. Pleiotropic constraint hampers the resolution of sexual antagonism in vertebrate gene expression. Am Nat. 2008:171(1):35–43. https://doi.org/10.1086/523954.
Markow TA. Perspective: female remating, operational sex ratio, and the arena of sexual selection in *Drosophila* species. Evolution. 2002:56(9):1725–1734. https://doi.org/10.1111/j.0014-3820.2002.tb00186.x.
Markow TA, Beall S, Matzkin LM. Egg size, embryonic development time and ovoviviparity in *Drosophila* species. J Evol Biol. 2009:22(2):430–434. https://doi.org/10.1111/j.1420-9101.2008.01649.x.
Markow TA, O'Grady PM. Evolutionary genetics of reproductive behavior in *Drosophila*: connecting the dots. Ann Rev Genet. 2005:39(1):263–291. https://doi.org/10.1146/annurev.genet.39.073003.112454.
Masalia RR, Bewick AJ, Burke JM. Connectivity in gene coexpression networks negatively correlates with rates of molecular evolution in flowering plants. PloS One. 2017:12(7):e0182289. https://doi.org/10.1371/journal.pone.0182289.
McDonald JH, Kreitman M. Adaptive protein evolution at the *Adh* locus in *Drosophila*. Nature. 1991:351(6328):652–654. https://doi.org/10.1038/351652a0.
Meisel RP. Towards a more nuanced understanding of the relationship between sex-biased gene expression and rates of protein-coding sequence evolution. Mol Biol Evol. 2011:28(6):1893–1900. https://doi.org/10.1093/molbev/msr010.
Mensch J, Serra F, Lavagnino NJ, Dopazo H, Hasson E. Positive selection in nucleoporins challenges constraints on early expressed genes in *Drosophila* development. Genome Biol Evol. 2013:5(11):2231–2241. https://doi.org/10.1093/gbe/evt156.
Miller PB, Obrik-Uloho OT, Phan MH, Medrano CL, Renier JS, Thayer JL, Wiessner G, Qazi MCB. The song of the old mother: reproductive senescence in female *Drosophila*. Fly (Austin). 2014:8(3):127–139. https://doi.org/10.4161/19336934.2014.969144.
Mitterboeck TF, Liu S, Adamowicz SJ, Fu J, Zhang R, Song W, Meusemann K, Zhou X. Positive and relaxed selection associated with flight evolution and loss in insect transcriptomes. Gigascience. 2017:6(10):1–14. https://doi.org/10.1093/gigascience/gix073.
Montague JR, Mangan RL, Starmer WT. Reproductive allocation in the Hawaiian Drosophilidae: egg size and number. Am Nat. 1981:118(6):865–871. https://doi.org/10.1086/283877.
Montgomery SH, Capellini I, Venditti C, Barton RA, Mundy NI. Adaptive evolution of four microcephaly genes and the evolution of brain size in anthropoid primates. Mol Biol Evol. 2011:28(1):625–638. https://doi.org/10.1093/molbev/msq237.
Munds RA, Titus CL, Moreira LAA, Eggert LS, Blomquist GE. Examining the molecular basis of coat color in a nocturnal primate family (*Lorisidae*). Ecol Evol. 2021:11(9):4442–4459. https://doi.org/10.1002/ece3.7338.
Murga-Moreno J, Coronado-Zamora M, Hervas S, Casillas S, Barbadilla A. iMKT: the integrative McDonald and Kreitman test. Nucl Acid Res. 2019:47(W1):W283–W288. https://doi.org/10.1093/nar/gkz372.
Nadeau NJ, Burke T, Mundy NI. Evolution of an avian pigmentation gene correlates with a measure of sexual selection. Proc: Biol Sci. 2007:274(1620):1807–1813. https://doi.org/10.1098/rspb.2007.0174.
Obbard DJ, Maclennan J, Kim K-W, Rambaut A, O'Grady PM, Jiggins FM. Estimating divergence dates and substitution rates in the *Drosophila* phylogeny. Mol Biol Evol. 2012:29(11):3459–3473. https://doi.org/10.1093/molbev/mss150.

Otto SP. Two steps forward, one step back: the pleiotropic effects of favoured alleles. Proc: Biol Sci. 2004:271(1540):705–714. https://doi.org/10.1098/rspb.2003.2635.
Proschel M, Zhang Z, Parsch J. Widespread adaptive evolution of *Drosophila* genes with sex-biased expression. Genetics. 2006:174(2):893–900. https://doi.org/10.1534/genetics.106.058008.
Ramm SA, Oliver PL, Ponting CP, Stockley P, Emes RD. Sexual selection and the adaptive evolution of mammalian ejaculate proteins. Mol Biol Evol. 2008:25(1):207–219. https://doi.org/10.1093/molbev/msm242.
Ranz JM, Castillo-Davis CI, Meiklejohn CD, Hartl DL. Sex-dependent gene expression and evolution of the *Drosophila* transcriptome. Science. 2003:300(5626):1742–1745. https://doi.org/10.1126/science.1085881.
R-Core-Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2022.
Rice WR. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. Nature. 1996:381(6579):232–234. https://doi.org/10.1038/381232a0.
Sahut-Barnola I, Dastugue B, Couderc JL. Terminal filament cell organization in the larval ovary of *Drosophila melanogaster*: ultrastructural observations and pattern of divisions. Roux's Arch Dev Biol. 1996:205(7-8):356–363. https://doi.org/10.1007/BF00377215.
Sarikaya DP, Belay AA, Ahuja A, Dorta A, Green DA, Extavour CG. The roles of cell size and cell number in determining ovariole number in *Drosophila*. Dev Biol. 2012:363(1):279–289. https://doi.org/10.1016/j.ydbio.2011.12.017.
Sarikaya DP, Church SH, Lagomarsino LP, Magnacca KN, Montgomery SL, Price DK, Kaneshiro KY, Extavour CG. Reproductive capacity evolves in response to ecology through common changes in cell number in Hawaiian *Drosophila*. Curr Biol. 2019:29(11):1877–-1884.e1876. https://doi.org/10.1016/j.cub.2019.04.063.
Sarikaya DP, Extavour CG. The hippo pathway regulates homeostatic growth of stem cell niche precursors in the *Drosophila* ovary. PLoS Genet. 2015:11(2):e1004962. https://doi.org/10.1371/journal.pgen.1004962.
Satija R, Farrell JA, Gennert D, Schier AF, Regev A. Spatial reconstruction of single-cell gene expression data. Nat Biotechnol. 2015:33(5):495–502. https://doi.org/10.1038/nbt.3192.
Sinclair CS, Lisa SF, Pischedda A. Does sexual experience affect the strength of male mate choice for high-quality females in *Drosophila melanogaster*? Ecol Evol. 2021:11(23):16981–16992. https://doi.org/10.1002/ece3.8334.
Singh A, Singh BN. Role of sexual selection in speciation in *Drosophila*. Genetica. 2014:142(1):23–41. https://doi.org/10.1007/s10709-013-9751-4.
Singh SR, Singh BN, Colorado Adoption P. Female remating in *Drosophila ananassae*: bidirectional selection for remating speed. Behav Genet. 2001:31(4):361–370. https://doi.org/10.1023/a:1012270318276.
Singh SR, Singh BN, Hoenigsberg HF. Female remating, sperm competition and sexual selection in *Drosophila*. Genet Mol Res. 2002:1:178–215.
Sirot LK, Wong A, Chapman T, Wolfner MF. Sexual conflict and seminal fluid proteins: a dynamic landscape of sexual interactions. Cold Spr Harbor Perspectives Biol. 2014:7(2):a017533. https://doi.org/10.1101/cshperspect.a017533.
Slaidina M, Banisch TU, Gupta S, Lehmann R. A single-cell atlas of the developing *Drosophila* ovary identifies follicle stem cell progenitors. Genes Dev. 2020:34(3-4):239–249. https://doi.org/10.1101/gad.330464.119.
Slaidina M, Gupta S, Banisch TU, Lehmann R. A single-cell atlas reveals unanticipated cell type complexity in *Drosophila* ovaries. Genome Res. 2021:31(10):1938–1951. https://doi.org/10.1101/gr.274340.120.
Song X, Zhu CH, Doan C, Xie T. Germline stem cells anchored by adherens junctions in the *Drosophila* ovary niches. Science. 2002:296(5574):1855–1857. https://doi.org/10.1126/science.1069871.
Srivastava M, Simakov O, Chapman J, Fahey B, Gauthier MEA, Mitros T, Richards GS, Conaco C, Dacre M, Hellsten U, et al. The Amphimedon queenslandica genome and the evolution of animal complexity. Nature. 2010:466(7307):720–726. https://doi.org/10.1038/nature09201.
Stanley CE Jr, Kulathinal RJ. flyDIVaS: a comparative genomics resource for *Drosophila* divergence and selection. G3 (Bethesda). 2016:6(8):2355–2363. https://doi.org/10.1534/g3.116.031138.
Starmer WT, Polak M, Pitnick S, McEvey SF, Barker JSF, Wolf LL. Phylogenetic, geographical, and temporal analysis of female reproductive trade-offs in Drosophilidae. Evol Biol. 2003:33:139–171. https://doi.org/10.1007/978-1-4757-5190-1_3.
Strasburg JL, Kane NC, Raduski AR, Bonin A, Michelmore R, Rieseberg LH. Effective population size is positively correlated with levels of adaptive divergence among annual sunflowers. Mol Biol Evol. 2011:28(5):1569–1580. https://doi.org/10.1093/molbev/msq270.
Sucena E, Stern DL. Divergence of larval morphology between Drosophila sechellia and its sibling species caused by cis-regulatory evolution of ovo/shaven-baby. Proc Nat Acad Sci U S A. 2000:97(9):4530–4534. https://doi.org/10.1073/pnas.97.9.4530.
Suvorov A, Kim BY, Wang J, Armstrong EE, Peede D, D'Agostino ERR, Price DK, Waddell P, Lang M, Courtier-Orgogozo V, et al. Widespread introgression across a phylogeny of 155 *Drosophila* genomes. Curr Biol. 2022:32(1):111–123 e115. https://doi.org/10.1016/j.cub.2021.10.052.
Swanson WJ, Vacquier VD. The rapid evolution of reproductive proteins. Nat Rev: Genet. 2002:3(2):137–144. https://doi.org/10.1038/nrg733.
Swanson WJ, Wong A, Wolfner MF, Aquadro CF. Evolutionary expressed sequence tag analysis of *Drosophila* female reproductive tracts identifies genes subjected to positive selection. Genetics. 2004:168(3):1457–1465. https://doi.org/10.1534/genetics.104.030478.
Symonds MRE, Blomberg SP. "Modern phylogenetic comparative methods and their application in evolutionary biology". In: Garamszegi LZ, editor. A primer on phylogenetic generalised least squares. Berlin Heidelberg: Springer-Verlag; 2014. p. 105–130.
Tamura K, Stecher G, Kumar S. MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol. 2021:38(7):3022–3027. https://doi.org/10.1093/molbev/msab120.
Tamura K, Subramanian S, Kumar S. Temporal patterns of fruit fly (*Drosophila*) evolution revealed by mutation clocks. Mol Biol Evol. 2004:21(1):36–44. https://doi.org/10.1093/molbev/msg236.
Tarikere S, Ylla G, Extavour CG. Distinct gene expression dynamics in germ line and somatic tissue during ovariole morphogenesis in *Drosophila melanogaster*. G3 (Bethesda). 2022:12(2):jkab305. https://doi.org/10.1093/g1093journal/jkab1305.
Tautz D, Domazet-Loso T. The evolutionary origin of orphan genes. Nat Rev Genet. 2011:12(10):692–702. https://doi.org/10.1038/nrg3053.
Tautz D, Neme R, Domazet-Loso T. Evolutionary origin of orphan genes. eLS. 2013. doi:10.1002/9780470015902.a0024601.
Thomas JA, Welch JJ, Lanfear R, Bromham L. A generation time effect on the rate of molecular evolution in invertebrates. Mol Biol Evol. 2010:27(5):1173–1180. https://doi.org/10.1093/molbev/msq009.
Treangen TJ, Rocha EP. Horizontal transfer, not duplication, drives the expansion of protein families in prokaryotes. PLoS Genet. 2011:7(1):e1001284. https://doi.org/10.1371/journal.pgen.1001284.
Veltsos P, Porcelli D, Fang Y, Cossins AR, Ritchie MG, Snook RR. Experimental sexual selection reveals rapid evolutionary

divergence in sex-specific transcriptomes and their interactions following mating. Mol Ecol. 2022:31(12):3374–3388. https://doi.org/10.1111/mec.16473.
Wagner GP, Zhang J. The pleiotropic structure of the genotype-phenotype map: the evolvability of complex organisms. Nat Rev: Genet. 2011:12(3):204–213. https://doi.org/10.1038/nrg2949.
Warnefors M, Kaessmann H. Evolution of the correlation between expression divergence and protein divergence in mammals. Genome Biol Evol. 2013:5(7):1324–1335. https://doi.org/10.1093/gbe/evt093.
Wayne ML, McIntyre LM. Combining mapping and arraying: an approach to candidate gene identification. Proc Nat Acad Sci U S A. 2002:99(23):14903–14906. https://doi.org/10.1073/pnas.222549199.
Whittle CA, Extavour CG. Refuting the hypothesis that the acquisition of germ plasm accelerates animal evolution. Nat Commun. 2016:7(1):12637. https://doi.org/10.1038/ncomms12637.
Whittle CA, Extavour CG. Selection shapes turnover and magnitude of sex-biased expression in *Drosophila* gonads. BMC Evol Biol. 2019:19(1):60. https://doi.org/10.1186/s12862-019-1377-4.
Whittle CA, Johannesson H. Evolutionary dynamics of sex-biased genes in a hermaphrodite fungus. Mol Biol Evol. 2013:30(11):2435–2446. https://doi.org/10.1093/molbev/mst143.
Whittle CA, Johnston MO. Broad-scale analysis contradicts the theory that generation time affects molecular evolutionary rates in plants. J Mol Evol. 2003:56(2):223–233. https://doi.org/10.1007/s00239-002-2395-0.
Whittle CA, Kulkarni A, Extavour CG. Evolutionary dynamics of sex-biased genes expressed in cricket brains and gonads. J Evol Biol. 2021:34(8):1188–1211. https://doi.org/10.1111/jeb.13889.
Whittle CA, Sun Y, Johannesson H. Genome-wide selection on codon usage at the population level in the fungal model organism *Neurospora crassa*. Mol Biol Evol. 2012:29(8):1975–1986. https://doi.org/10.1093/molbev/mss065.
Wigby S, Brown NC, Allen SE, Misra S, Sitnik JL, Sepil I, Clark AG, Wolfner MF. The *Drosophila* seminal proteome and its role in post-copulatory sexual selection. Philos Trans R Soc Lond B Biol Sci. 2020:375(1813):20200072. https://doi.org/10.1098/rstb.2020.0072.
Wlasiuk G, Nachman MW. Promiscuity and the rate of molecular evolution at primate immunity genes. Evolution. 2010:64(8):2204–2220. https://doi.org/10.1111/j.1558-5646.2010.00989.x.
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. Genome-wide midrange transcription profiles reveal expression level relationships in human tissue specification. Bioinformatics. 2005:21(5):650–659. https://doi.org/10.1093/bioinformatics/bti042.
Yang Z. PAML: a program package for phylogenetic analysis by maximum likelihood. Comput App Biosci. 1997:13(5):555–556. https://doi.org/10.1093/bioinformatics/13.5.555.
Yang Z. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. Mol Biol Evol. 1998:15(5):568–573. https://doi.org/10.1093/oxfordjournals.molbev.a025957.
Yang Z. PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol. 2007:24(8):1586–1591. https://doi.org/10.1093/molbev/msm088.
Yang Z, Nielsen R. Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. Mol Biol Evol. 2002:19(6):908–917. https://doi.org/10.1093/oxfordjournals.molbev.a004148.
Yasothornsrikul S, Davis WJ, Cramer G, Kimbrell DA, Dearolf CR. Viking: identification and characterization of a second type IV collagen in *Drosophila*. Gene. 1997:198(1-2):17–25. https://doi.org/10.1016/s0378-1119(97)00274-6.
Zhang J, Nielsen R, Yang Z. Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. Mol Biol Evol. 2005:22(12):2472–2479. https://doi.org/10.1093/molbev/msi237.

Associate editor: Andrea Betancourt