Report

Current Biology

Reproductive Capacity Evolves in Response to Ecology through Common Changes in Cell Number in Hawaiian *Drosophila*

Highlights

- Ecology and development predict fecundity evolution in Hawaiian *Drosophila*
- Where Hawaiian flies lay their eggs influences evolution of reproductive capacity
- Allometric relationship between body and ovary size differs by habitat type
- Changes in somatic gonad cell number explain convergent ovariole number evolution

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In Brief

Organisms leaving more offspring likely have higher fitness. Sarikaya et al. use the adaptive radiation of Hawaiian *Drosophila* to investigate the evolution of fecundity. They find that habitat shifts played a strong role and identify a developmental process that underlies evolutionary change in ovarian development and impacts egg-laying capacity.

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Report

Reproductive Capacity Evolves in Response to Ecology through Common Changes in Cell Number in Hawaiian *Drosophila*

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SUMMARY

Lifetime reproductive capacity is a critical fitness component. In insects, female reproductive capacity is largely determined by the number of ovarioles, the egg-producing subunits of the ovary [e.g., 1]. Recent work has provided insights into ovariole number regulation in *Drosophila melanogaster*. However, whether mechanisms discovered under laboratory conditions explain evolutionary variation in natural populations is an outstanding question. We investigated potential effects of ecology on the developmental processes underlying ovariole number evolution among Hawaiian *Drosophila*, a large adaptive radiation wherein the highest and lowest ovariole numbers of the family have evolved within 25 million years. Previous studies proposed that ovariole number correlated with oviposition substrate [2–4] but sampled largely one clade of these flies and were limited by a provisional phylogeny and the available comparative methods. We test this hypothesis by applying phylogenetic modeling to an expanded sampling of ovariole numbers and substrate types and show support for these predictions across all major groups of Hawaiian *Drosophila*, wherein ovariole number variation is best explained by adaptation to specific substrates. Furthermore, we show that oviposition substrate evolution is linked to changes in the allometric relationship between body size and ovariole number. Finally, we provide evidence that the major changes in ovarian cell number that regulate *D. melanogaster* ovariole number also regulate ovariole number in Hawaiian drosophilids. Thus, we provide evidence that this remarkable adaptive radiation is linked to evolutionary changes in a key reproductive trait regulated at least partly by variation in the same developmental parameters that operate in the model species *D. melanogaster*.

RESULTS AND DISCUSSION

Adult Reproductive Traits of Hawaiian *Drosophila*

We measured three adult traits relevant to reproductive capacity (body size, ovariole number, and egg volume) from field-collected females, lab-reared first filial generation (F1) offspring of field-collected females, and females from laboratory strains (Figure 1; Table S1). Species identities of field-collected females were assigned based on morphological keys or DNA barcoding (Tables S2 and S3). All traits ranged over an order of magnitude within Hawaiian *Drosophila*: body size ranged from 0.71 mm for *Scaptomyza devexa* to 3.12 mm for *D. melanocephala*; ovariole number per female ranged from two for *S. caliginosa* to 88.5 for *D. melanocephala*; and egg volume ranged from 0.01 μm^3 for the *Scaptomyza (Bunostoma) spp.* group (*S. palmae*/*S. anomala*) to 0.2 μm^3 for *D. adunca*, highlighting the diversity of life history traits in Hawaiian *Drosophila*.

Within the *melanogaster* subgroup species, species-specific differences in ovariole number are largely heritable [e.g., 5]. To test whether this is also the case in Hawaiian *Drosophila*, we compared ovariole numbers in wild-caught females and their lab-reared F1 offspring, across five species with different egg-laying substrates. We observed no significant differences between the ovariole numbers of these two generations, regardless of natural substrate (Figure S1), indicating that species-specific differences in ovariole number are also strongly genetically determined in Hawaiian *Drosophila*.

Larval Ecology Influences Ovariole Number Evolution

Major shifts in ovariole number have often been attributed to changes in life history strategies. Ovoviviparity is often correlated with reduced ovariole number in Diptera [6], suggesting that increased parental investment could be linked to reduced

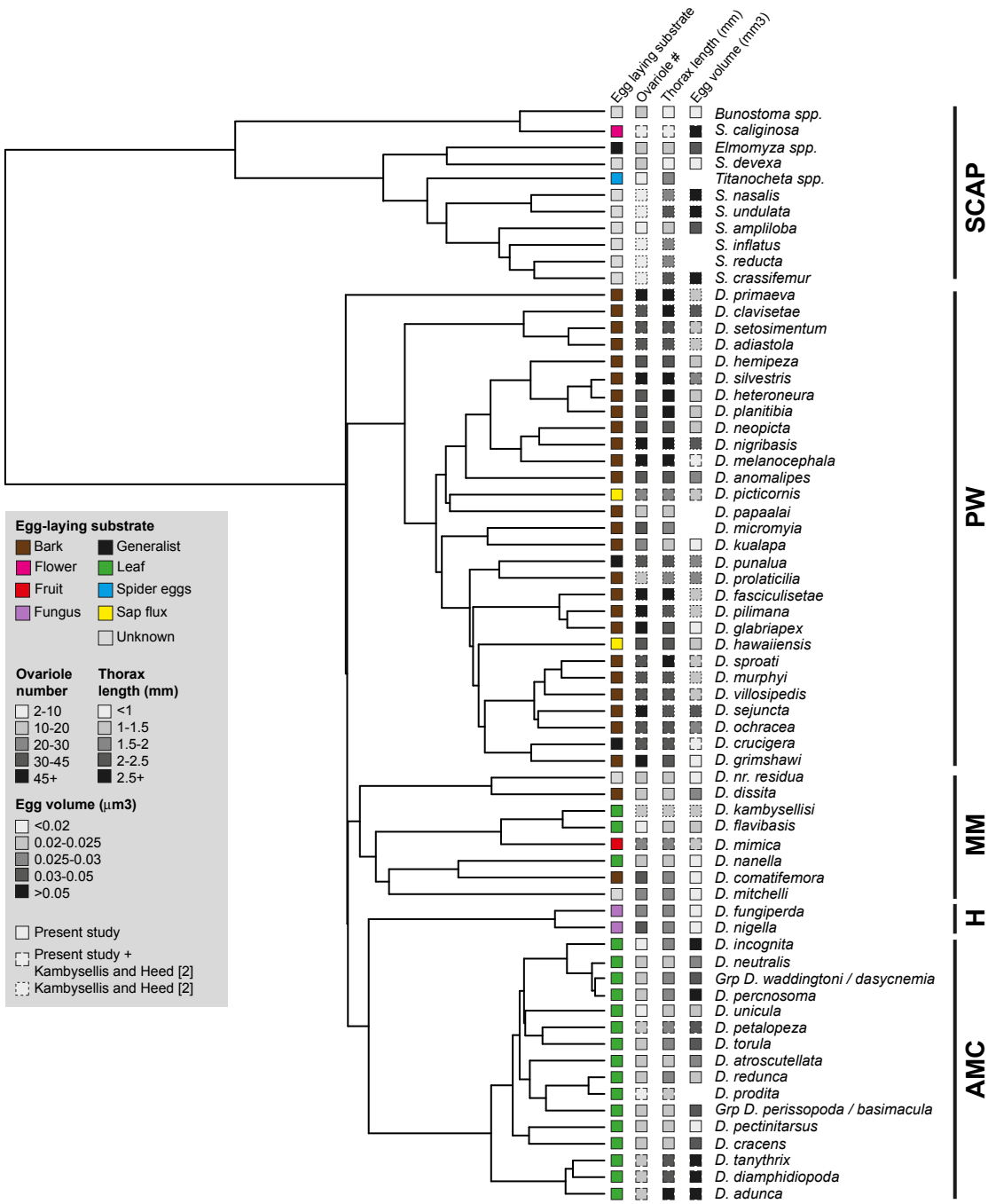

Figure 1. Reproductive and Ecological Traits of Hawaiian *Drosophila* in Phylogenetic Context

Compiled adult life history traits (grayscale gradients) collected herein and by Kambysellis and Heed [2] mapped onto a phylogeny of Hawaiian *Drosophila* constructed from available mitochondrial and nuclear genes. Egg-laying substrate of each species is indicated by colored boxes: bark (brown), generalist (black), sap flux (yellow), leaf (green), fungus (purple), fruit (red), spider eggs (blue), flowers (pink), and unknown (gray). Boxes with solid outlines denote data collected in the present study; boxes outlined with four notches denote data represented in our data and those of Kambysellis and Heed [2]; and boxes with dotted outlines denote data represented only in Kambysellis and Heed [2]. Missing boxes indicate data points either that were not previously reported [2] or that we were unable to obtain from field-caught samples. Black lines at right delineate the five major groups of Hawaiian *Drosophila* as follows: SCAP, *Scaptomyza*; PW, picture wing; MM, modified mouthparts; H, haleakalae; AMC, antopocerus-modified tarsus-ciliated tarsus.

See also Figure S1 and Tables S1, S2, and S3.

fecundity in these flies, as observed in other animals [7]. In the *Drosophila melanogaster* subgroup, previous studies have suggested that reproductive strategies and ovariole number evolve in response to oviposition or larval nutrition substrate [8, 9]. Most *melanogaster* subgroup species are generalists that oviposit on a variety of decaying fruits, and their mean ovariole number ranges from 18 to 43 per female [10]. Lachaise [9] proposed that the high ovariole number observed in the generalists *D. melanogaster* and *D. simulans* may be driven by the frequent oviposition opportunities available to generalist species compared to specialists. In contrast, *D. erecta* and *D. sechellia* are specialists on *Pandanus* fruit and the toxic *Morinda* fruit, respectively [11, 12], and *D. sechellia* has the lowest reported ovariole number of the group [13]. This reduction in ovariole number has been hypothesized to be the result of increased egg size as an adaptation to feeding on the toxic *Morinda* [14] or to be due to lower insulin signaling levels evolved in response to the relatively constant nutritional input provided by substrate specialization [15]. However, the *melanogaster* subgroup is not well suited for a broader understanding of ovariole number evolution, as most species share similar oviposition substrates (i.e., rotting fruit), and there are few independent instances of the evolution of specialists.

In contrast, Hawaiian *Drosophila* have evolved to specialize on a variety of oviposition substrates, including decaying flowers, leaves, fungi, sap fluxes, and the bark of native plants, as well as the eggs of native spiders [16]. Moreover, these flies exhibit the most extreme interspecies range of ovariole number reported in the genus, ranging from two to 101 per female [2]. Hawaiian *Drosophila* have undergone rapid island radiation from a common ancestor in the past 25 million years, leading to approximately 1,000 extant species [17, 18]. Most of the species diversity of Hawaiian *Drosophila* is spread across five monophyletic species groups that share genetic, morphological, and ecological similarities and rely on different oviposition substrates [18–21], as follows (Figure 1): *Scaptomyza* are small species that lay eggs on leaves, flowers, and fruits, and only approximately one third of *Scaptomyza* species are reported to be generalists. Picture wing (PW) species are larger species with striking pigment patterns on their wings. PW species primarily lay eggs on decaying bark or branches of native trees, though some specialize on sap fluxes [16]. Modified mouthpart (MM) species, which have male-specific modifications on mouthparts used during mating [22], have the largest range of egg-laying substrates, including bark, leaves, fruit, and sap fluxes. However, among MM species, the predominant egg-laying substrate is bark, and those MM species that are not bark breeders are mostly generalists and leaf specialists [16]. Haleakalae species are darkly pigmented flies that only lay eggs on fungi. Lastly, most antopocerus-modified tarsus-ciliated tarsus (AMC) species are leaf breeders, though there are a few exceptions that have evolved bark breeding [18].

Within the five major clades of Hawaiian *Drosophila*, ovariole number is highest in the PW species (up to 88 per female) and lowest in *Scaptomyza* and AMC species (as few as two per female) [2]. Dramatic differences in ovariole number between species were historically suggested to be associated with evolutionary shifts between oviposition substrates [2]. Subsequent studies [3, 4] found significant associations between ovariole numbers and some substrate types, in support of the earlier predictions. However, these studies either lacked a phylogenetic framework [2] or incorporated phylogenies including largely PW species [3, 4] that have since been improved upon with expanded taxon and locus sampling [18, 19, 21, 23].

Using an updated phylogenetic framework (see STAR Methods) and expanding taxon sampling across all major groups of Hawaiian *Drosophila*, we tested this hypothesis by comparing the fit of evolutionary models of ovariole number that accounted for ecologically driven evolution to those that did not. We combined original observations reported in the present study with data previously published by Kambysellis and Heed [2] (see STAR Methods). The combined dataset nearly doubles the number of species previously studied, includes both specialist species (which oviposit on bark, sap flux, leaves, fungus, fruit, flowers, or spider eggs) and generalist species (which oviposit on multiple decaying substrates), and adds new substrate types (spider eggs and flowers). We compared the fit of five models to our data, two of which—(1) Brownian motion (BM) and (2) an Ornstein-Uhlenbeck model with a shared optimum for all species (OU1)—did not take into account the oviposition substrate, and three of which were nested ecological models based on alternative methods of substrate classification: (3) the OU2 model assumed two states, bark breeders and all other species, to test previous suggestions that bark breeding may drive evolution of ovariole number [3, 4]; (4) the OU3 model assumed three states, *Scaptomyza* specialists on spider eggs and flowers, bark breeders, and species using any other substrate, to test hypotheses that substrates influence ovariole number evolution because of their differences in carrying capacity and field predictability [2, 8]; and (5) the OU8 model categorized each oviposition substrate separately. These five models were fit over 100 trees sampled from the posterior distribution of a Bayesian phylogenetic analysis to account for phylogenetic uncertainty.

We found that models accounting for larval ecology explained the ovariole number diversification in Hawaiian *Drosophila* (Figure 2A) better than those that did not. Comparing the three ecological models, we found that the three-state model (OU3), which accounted for both bark breeders and *Scaptomyza* specialists, was supported as the best-fit model across a majority of trees for ovariole number (ΔAICc [Akaike information criterion] > 2 as compared to OU2 and OU8 models; Data S1). Estimated theta values for the OU3 model showed that bark breeders have more ovarioles than species that oviposit on other substrates, suggesting that evolution of higher ovariole numbers accompanied the transition to bark breeding from likely non-bark breeding ancestors (Figures 2B and 2C; Data S1), consistent with earlier hypotheses [3, 4]. In contrast, *Scaptomyza* species may have experienced a dramatic decrease in ovariole number as they independently specialized on spider eggs and flowers (Figure 2C). Taken together, these results confirm and extend previous work in two important ways. First, they support the suggestions of Kambysellis and colleagues [2–4] that shifts in oviposition substrate may have contributed to the evolution of diverse ovariole numbers. Further, these results account for phylogenetic history, using robust comparative methods, and expand the previous taxon sampling to show that this trend applies not only to the PW flies that were most heavily studied

A AICc values

	AICc	ΔAICc	w(AIC)
BM	86.26	5.91	0.04
OU1	88.41	8.06	0.01
OU2	84.84	4.49	0.1
OU3	**80.35**	**0**	**0.77**
OU8	84.42	4.07	0.08

Figure 2. Different Ecological States Tested for OU Analysis

(A) Comparison of Akaike information criterion (AICc) and weighted AICc values for models testing the relationship between oviposition substrate and ovariole number. Values are for model fit of Brownian motion (BM) and the Ornstein-Uhlenbeck model with one optimum (OU1) or with multiple optima (OUMs) with different combinations of oviposition substrate categories, calculated with the R package OUwie v.1.48 [24]. Oviposition substrates were categorized as follows: OU2 categorizes species that lay eggs on bark and non-bark; OU3 categorizes species into bark breeder, spider egg/flower breeder, and other; and OU8 categorizes each species according to the eight oviposition substrates represented (bark, flower, spider egg, fruit, leaf, generalist, fungus, and sap flux). Models were tested over 100 posterior distribution BEAST trees using nuclear and mitochondrial gene sequences. Bold indicates the best supported model.

(B) A two-state model (OU2) of bark breeders (brown) and non-bark breeders (white).

(C) Three-state model (OU3) that codes bark breeders (brown), spider egg and flower breeders (blue), and other oviposition substrates (white).

(D) Eight-state model (OU8) that codes each egg-laying substrate separately, color coded as in Figure 1. Pie charts show the maximum likelihood ancestral state estimates at each node, calculated with the rayDISC function in the R package corHMM,v.1.18 [25].

See also Data S1.

previously [3, 4] but also across all major groups of the adaptive radiation of Hawaiian *Drosophila*.

Evolution of Specialist Habitats Changes Allometry of Reproductive Traits

Across animals, potential fecundity sometimes correlates positively with body size [e.g., 26, 27]. The range of Hawaiian *Drosophila* body sizes is greater than that of other members of the genus, spanning an order of magnitude (Table S1). To determine whether changes in allometric growth might underlie reproductive trait evolution, we analyzed the allometric ratio of such traits using a phylogenetic least-squares (PGLS) analysis and thorax volume (thorax cubed length) as a proxy for body size. We found that, across all Hawaiian *Drosophila*, thorax volume was significantly positively correlated with both ovariole number (Figure 3A; Table S4) and egg volume (Figure 3B; Table S4). However, individual species groups show differences in trends for allometric ratios of reproductive traits. In PW and MM species, body size is correlated positively with ovariole number (Figures 3A1 and 3A2) but not with egg volume (Figures 3B1 and 3B2). In contrast, AMC and *Scaptomyza* species have a positive correlation with body size and egg volume (Figures 3B3 and 3B4) but not ovariole number (Figures 3A3 and 3A4). For PW, MM, and AMC, there is a negative correlation between ovariole number and proportional egg size (Tables S2 and S4), and there is a negative correlation between ovariole number and egg volume in AMC and *Scaptomyza* (Table S4).

We note that these trends are associated with differences in life history strategies between groups. In PW and MM group species, ovariole number increases with increasing body size (Figures 3A1 and 3A2): PW species are primarily bark breeders that oviposit eggs in clutches of up to 100 eggs [2], and about half of MM species are bark breeders [16]. Our analysis suggests that bark breeding was ancestral to both species groups (Figure 2C). In contrast, the AMC and *Scaptomyza* species groups, in which ovariole number and body size are decoupled (Figures 3A3 and 3A4), contain very few bark breeding species and, instead, have evolved to use a variety of different substrates. AMC group species are primarily leaf breeders. *Scaptomyza* species include specialists on leaves, flowers, fruits, and spider eggs, as well as host plant specialist species that oviposit on all parts of the plant, and fewer than 5% of *Scaptomyza* are bark specialists [16]. In sum, while a positive correlation between body size and fecundity is commonly posited in egg-laying animals [e.g., 26, 27], we did not find universal support for this trend across Hawaiian *Drosophila*. This is consistent with previous studies on Diptera, wherein trends toward higher fecundity or ovariole number in larger animals observed within species [26] contrast with between-species differences in ovariole number that do not always correlate with body size [9, 29].

Larval Ovary Somatic Cell Number Determines Ovariole Number

Ovariole number is determined during larval development, when a specific group of cells called terminal filament cells (TFCs) form stacks, called terminal filaments (TFs), which serve as the beginning point of each ovariole [30]. While in at least some insects, TF destruction during pupal stages can also contribute to final ovariole number [31], TF formation appears to be a prevalent mechanism determining ovariole number between and within *Drosophila* species [32–34]. This then leads to the question of what developmental processes determine how many TFs will form. We previously identified two cell number and cell behavior parameters that can alter TF number and, thus, ovariole number: (1) changes in TFC number per TF and (2) changes in total TFC number [32]. To determine whether the same developmental

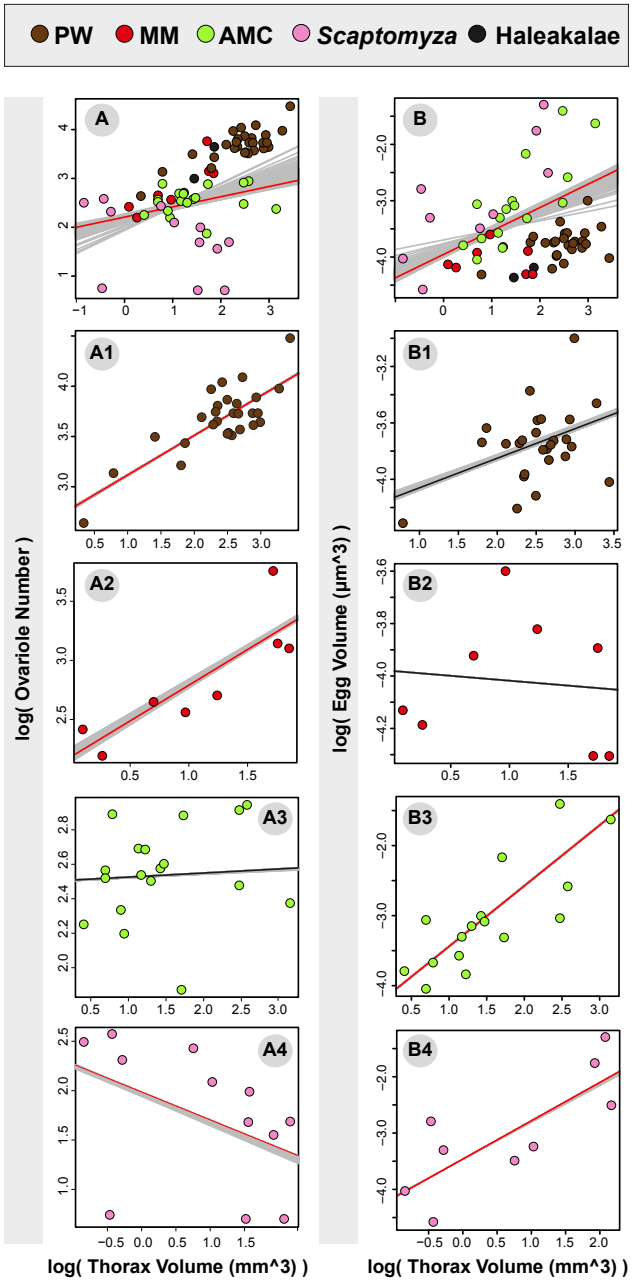

Figure 3. Allometric Relationship between Life History Traits in Hawaiian *Drosophila*

Scatterplots of log-transformed adult measurements with phylogenetically transformed trend lines generated by averaging runs from PGLS analysis across 100 posterior distribution BEAST trees, performed with the R package nlme v.3.1-121 [28]. Trend line of the consensus tree is denoted in red when there was a significant relationship between the two traits and black when PGLS analysis did not support a significant relationship (Data S1).

(A–A4) Ovariole number plotted against thorax volume (in cubed millimeters) in (A) all specimens, (A1) PW, (A2) MM, (A3) AMC, and (A4) *Scaptomyza*.

(B–B4) Egg volume (in cubed millimeters) plotted against thorax volume (in cubed millimeters) in (B) all specimens, (B1) PW, (B2) MM, (B3) AMC, and (B4) *Scaptomyza*.

See also Table S4.

processes that regulate ovariole number in laboratory populations also underlie the evolution of ovariole number in natural populations, we measured TF and TFC numbers in the developing larval ovaries of Hawaiian *Drosophila*. Our analysis of 12 species representing four of the major Hawaiian *Drosophila* species groups showed that, even over a range of ovariole numbers spanning an order of magnitude (Figure 4; Table S5), larval TF number per ovary essentially corresponded to adult ovariole number per ovary (Table S5). Although TFC number per TF varied somewhat between species (Figure 4A; Table S5), PGLS analysis showed no correlation between TFC number per TF and total TF number (Table S6). In contrast, average total TFC number was strongly positively correlated with TF number (Figure 4B; Tables S5 and S6), suggesting that, as in laboratory populations of *D. melanogaster*, changes in TFC number underlie ovariole number evolution in Hawaiian *Drosophila*.

The developmental processes underlying ovariole number evolution are particularly interesting in light of the allometric changes in Hawaiian *Drosophila* species groups. There has been some debate as to whether allometry constrains or facilitates adaptive evolution [e.g., 35]. In Hawaiian *Drosophila*, the allometric relationship between two important female reproductive traits—ovariole number and egg size—was coupled to body size in different groups in different ways: when ovariole number was coupled with body size, egg size was not, and vice versa (Figure 3). These trends were associated with bark breeding in the PW and MM groups, where ovariole number was coupled with body size (Figures 1 and 2). While the phenotypic integration of ovariole number and egg volume appears tightly regulated across insects [36], the coupling of ovariole number to body size appears more flexible in Hawaiian *Drosophila*, suggesting that, in this context, heritable changes in allometry may contribute to adaptive evolution.

Ovariole number is highly polygenic [e.g., 5, 37] and is regulated by both intrinsic and extrinsic growth factors. Many of these genes, including Hippo signaling, ecdysone, and insulin-like peptides, are pleiotropic and can also regulate body size [e.g., 15, 38–40]. Thus, we propose that the mechanistic basis for evolutionary change of ovariole number on different substrates may be changes in the relative influence of nutritionally regulated circulating growth factors on the one hand, and cell-autonomous growth on the other hand, on ovarian development during larval and pupal stages. For example, we speculate that, on certain substrates, the larval ovary may become less sensitive to nutritionally mediated growth factors by evolving lower expression levels of growth factor receptors and relying more on tissue-specific growth factors, which could include local insulin release or cell proliferation pathways such as Hippo signaling.

In summary, by combining phylogenetic comparative methods with comparative developmental analyses of both wild-caught flies and laboratory strains, we have identified potential mechanisms of evolutionary change in ovariole number operating at three levels of biological organization. First, evolutionary shifts in ecological niche can predict the dramatic differences in ovariole number in Hawaiian *Drosophila*. Second, whether adult body size is coupled with ovariole number or egg volume differs between species groups with different oviposition substrates, suggesting that the allometric growth relationships between these traits evolve dynamically. Finally, changes

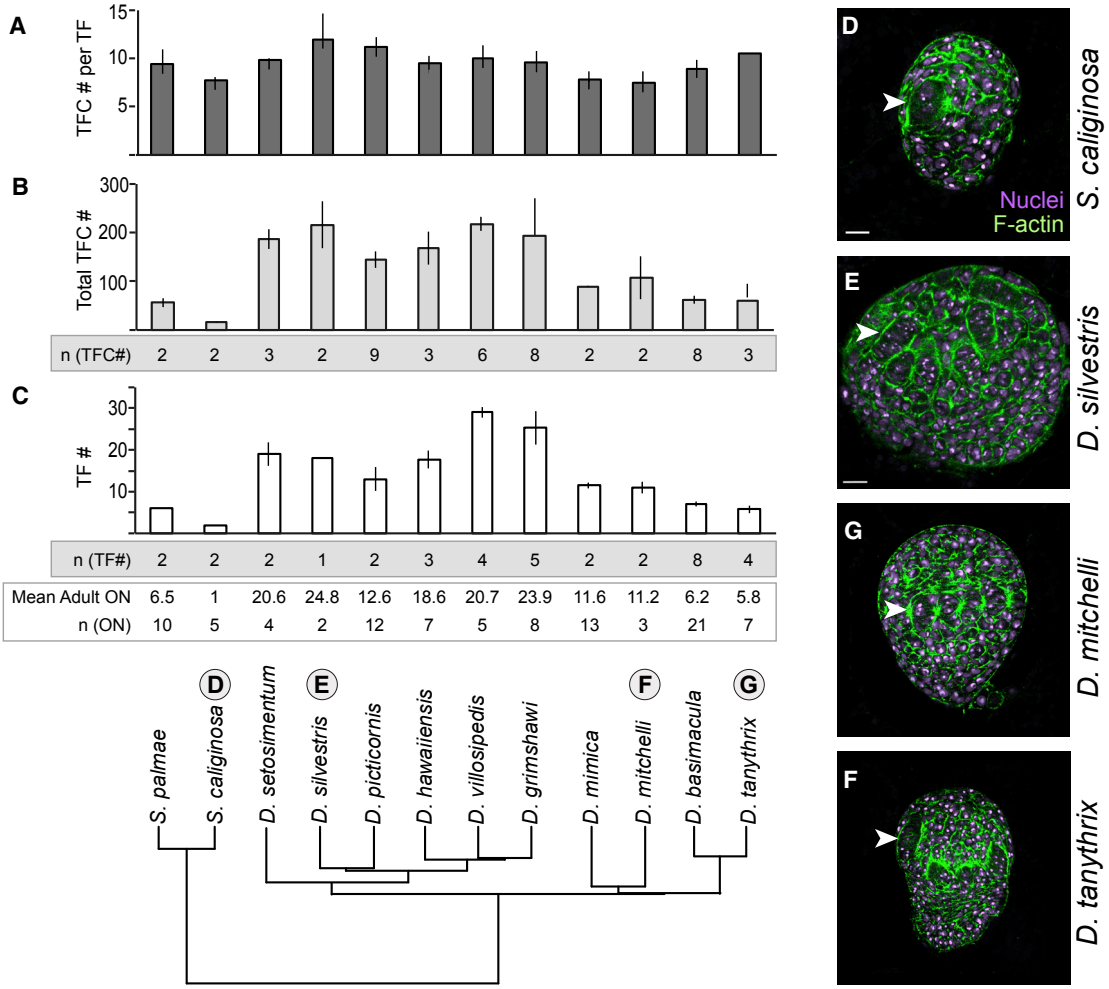

Figure 4. Terminal Filament Cell Number Predicts Terminal Filament Number in Hawaiian Drosophilids

(A–C) Bar graphs for (A) terminal filament cell (TFC) number per terminal filament (TF), (B) total TFC number per larval ovary, and (C) TF number per larval ovary representing the mean and SD of each parameter, as well as the phylogenetic relationship between the species shown (bottom). Gray panel below (B) and (C) indicates sample sizes used to determine total TFC number and mean TF number, respectively (n = number of ovaries from lab-born larvae of wild-caught females; Table S5). Gray-outlined box below (C) indicates mean ovariole number (ON) per adult ovary (Table S1) and sample sizes used to determine mean ovariole number (n = number of wild-caught adult females; Table S1) for each species.

(D–F) Late third instar larval ovaries stained for nuclei (purple) and F-actin (green) for (D) *S. caliginosa* (flower breeder), (E) *D. silvestris* (bark breeder), (G) *D. mitchelli* (egg-laying substrate unknown), and (F) *D. tanythrix* (leaf breeder). White arrowheads indicate TF structures in the ovary.

See also Tables S5 and S6.

in ovariole number from 2 to 60 per individual can be explained by changes in total TFC number, suggesting that ovariole number diversity evolves through the same developmental processes, regardless of the specific ecological constraints or selective pressures. Thus, by integrating ecology, organismal growth, and cell behavior during development to understand the evolution of ovariole number, this work connects the ultimate and proximate mechanisms of evolutionary change in reproductive capacity.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.04.063.

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AUTHOR CONTRIBUTIONS

D.P.S. conceived of the study, obtained funding for the study, collected flies from the field, identified fly species, designed and executed morphological and developmental experiments, analyzed and interpreted results, and wrote the paper. S.H.C. and L.P.L. performed phylogenetic analyses and helped revise the paper. K.N.M. and S.L.M. collected flies from the field. K.Y.K. and D.K.P. assisted with lab manipulation of wild-caught flies and helped revise the paper. C.G.E. conceived of the study, provided funding for the study, interpreted results, and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-Engrailed/Invected	Developmental Studies Hybridoma Bank	4D9; RRID: AB_528224
Biological Samples		
DNA from field collected Hawaiian *Drosophila*	This study	Table S2
Chemicals, Peptides, and Recombinant Proteins		
FITC-conjugated Phalloidin	Sigma	Cat# P5282
Hoechst 33342	Sigma	Cat# B2261
Vectashield Anti-fade mounting medium	Vector Labs	Cat# H-1000
Dynazyme DNA Polymerase	Thermo Scientific	Cat#: F501
ExoSAP-IT	Thermo Scientific	Cat#: 78201
Critical Commercial Assays		
DNeasy Blood and Tissue DNA extraction kit	QIAGEN	Cat#: 69506
Deposited Data		
COI sequences from picture wing species identified using morphological keys	This study	GenBank: MK276992–MK277193
Mitochondrial sequences from field caught Hawaiian *Drosophila* for DNA barcoding	This study	https://github.com/shchurch/hawaiian_Drosophila_ovaries_2018/tree/master/sequence_data_methods/sarikaya2018_original_sequence_data
RAxML phylogenetic trees for species delineation using DNA barcoding	This study	https://github.com/shchurch/hawaiian_Drosophila_ovaries_2018/tree/master/sequence_data_methods/species_delineation_gene_trees
Adult and larval phenotypic data	This study	https://github.com/shchurch/hawaiian_Drosophila_ovaries_2018/blob/master/data.txt
Hawaiian *Drosophila* phylogeny	This study using sequences from [18, 19, 21, 23]	https://github.com/shchurch/hawaiian_Drosophila_ovaries_2018
Oligonucleotides		
COI F: ATT CAA CCA ATC ATA AAG ATA TTG G	[18]	N/A
COI R: TAA ACT TCT GGA TGT CCA AAA AAT CA	[18]	N/A
COII F: ATG GCA GAT TAG TGC AAT GG	[18]	N/A
COII R: GTT TAA GAG ACC AGT ACT TG	[18]	N/A
ND2 F: AGCTATTGGGTTCAGACCCC	[18]	N/A
ND2 R: GAAGTTTGGTTTAAACCTCC	[18]	N/A
Software and Algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/
4Peaks	Nucleobytes	https://nucleobytes.com/4peaks/index.html
Phyutility v2.2.6	[41]	https://github.com/blackrim/phyutility
MAFFT v7.130b	[42]	https://mafft.cbrc.jp/alignment/software/
Gblocks v091b	[43]	http://molevol.cmima.csic.es/castresana/Gblocks/Gblocks_documentation.html
PartitionFinder v1.1.1_Mac	[44]	http://www.robertlanfear.com/partitionfinder/
RAxML v8.2.3	[45]	https://cme.h-its.org/exelixis/web/software/raxml/index.html
BEAST v2.3.2	[46, 47]	http://beast.community/
CIPRES cluster	Cyberinfrastructure for Phylogenetic Research	http://www.phylo.org/

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
AWTY	[48]	http://evomics.org/resources/software/molecular-evolution-software/awty/
Tracer	[49]	http://www.beast2.org/tracer-2/
TreeAnnotator	[50]	http://beast.community/treeannotator
R v3.2.0	[51]	https://www.r-project.org/
Nlme v3.1-121	[28]	https://cran.r-project.org/web/packages/nlme/
Ape v3.3	[52, 53]	https://cran.r-project.org/web/packages/ape/
corHMM v1.18	[25]	https://cran.r-project.org/web/packages/corHMM/
OUwie v1.18	[24]	https://cran.r-project.org/web/packages/OUwie/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Cassandra G. Extavour (extavour@oeb.harvard.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Field collections of Hawaiian *Drosophila*

Field collections of Hawaiian *Drosophila* were conducted under the Department of Land and Natural Resources of Hawai'i native invertebrate scientific collection permits FHM14-305, FHM14-353, and HAVO-2013-SCI-0002. Collections were made at the Koke'e State Park and Kui'a Natural area reserve (NAR) on Kauai, West Maui Forest Reserve, Makawao Forest Reserve, and the Nature Conservancy's Waikamoi Preserve on Maui, and Hawaii Volcanoes National Park and Upper Waiakea Forest Reserve on Hawai'i island. Flies were collected by aspirating flies from traps or sponges containing fermenting fruit and fungi, or by sweeping and sorting leaf litter in forests.

Husbandry of Hawaiian *Drosophila*

Field-caught females were maintained on yeast-less Wheeler-Clayton medium or *Drosophila* standard laboratory medium at 18°C at 80% humidity. Each vial contained a piece of tissue paper (Kleenex) moistened with distilled water that was steeped with bark of *Clermontia spp*. at ambient temperature for several days to stimulate oviposition [54]. In addition, AMC species were supplemented with pieces of decaying *Cheirodendron* leaves, and *S. caliginosa* was supplemented with morning glory flowers (*Ipomea acuminata*), both of which were collected from the field and frozen overnight to eliminate mites.

Larvae of picture wing subgroup and antopocerus species of the AMC subgroup have longer development times than the other species studied herein, and larvae of these species were fed additional food, which was made as follows: 6g of Agar and 225mL distilled water were mixed in a 1L beaker and microwaved for two minutes. 60 g cornmeal, 6.6g roasted soybean meal and 7.5g brewer's yeast were mixed, blended and added to the beaker along with an additional 300mL distilled water, and mixed with a spoon. Lastly, three tablespoons of Karo light corn syrup and one tablespoon of unsulfured molasses was added to the mix, and the mixture was microwaved for three minutes. Food was mixed every minute during microwaving until the mixture was close to boiling point and started to rise up within the beaker. The beaker containing hot food was placed at room temperature until the mixture was warm enough to touch. 3mL of propionic acid and 3mL of 99% ethanol were added, and the solidified food was stored at 4°C. The solidified food was mixed with a small quantity of water to soften the consistency before being used to feed larvae.

Non-picture wing Hawaiian *Drosophila* species pupariated on the side of the glass vials, and hatched F1 offspring were transferred into new vials. Larvae of the picture wing subgroup species pupate in the soil. To accommodate this behavior, food vials with wandering picture wing larvae were placed in a large jar containing 1-2cm of moist sand at the bottom. A piece of cloth or paper towel held in place using a rubber band was used close the opening of each jar. Larvae migrated from the vials to the sand to pupariate, and thus adults emerged from the sand, and were aspirated out of the jar into a fresh adult food vial.

The effect of larval substrate on ovariole number in lab-reared and wild Hawaiian *Drosophila*

Evolutionary modeling analysis showed that ovariole number in Hawaiian *Drosophila* is best explained by evolutionary forces related to egg-laying substrate. Specifically, the three-state model that we tested (OU3), which distinguishes between bark breeding, the specialist substrates of *Scaptomyza* specialists, and other substrates, was the best fit for our ovariole number data across a majority of trees for ovariole number ($\Delta AICc > 2$ as compared to OU2 and OU8 models). The Brownian motion (BM), one-state model (OU1) and two-state (OU2) model lacked support as compared to the OU3 model ($\Delta AICc > 2$). Along with strong support for the OU3 model, we obtained occasional support for the full eight state model (OU8) (Data S1). It is possible that the limited sample size for some substrate categories in this model contributed to the poor fit of OU8 to our data. Further studies with deeper sampling to obtain increased

representation of non-bark oviposition substrates will be needed clarify the extent to which finer distinctions between specific specialist substrates may contribute to adaptive changes in ovariole number.

These results were largely unchanged when comparing models using an alternative topology generated from mtDNA sequences (Data S1), with all analyses supporting a role for ecology in driving trait evolution. Since five out of the 66 species represented in the analysis were categorized into species groups rather than a single species based on the DNA barcoding, we assigned two different species IDs and ran the OU analysis to address whether group assignments had an impact on our analysis. Assigning IDs to closely related species within the group did not alter the results (Data S1). Similarly, results were unchanged when only the data collected for this study were considered, excluding data previously reported by Kambysellis and Heed [2] (Data S1).

Here we note some of the difficulties faced by researchers wishing to rigorously and thoroughly account for oviposition substrate in these analyses. Species keys are not available for females of most non-PW Hawaiian *Drosophila* species, and DNA barcode data that were used to identify the species were not available for many samples that were collected. Since Haleakala species were difficult to key, most samples from this group were excluded from the analysis. Further, presence of specific Hawaiian *Drosophila* species in the field can be unpredictable, and difficulty in encountering them during field work is increasingly compounded by the declining numbers of endemic species in Hawai'i. For example, sap flux specialists have been documented to exist in the PW, MM, and AMC groups [16], but we were only able to collect data from two PW sap flux breeders, one from the field (*D. picticornis*) and another from a laboratory line (*D. hawaiiensis*). Lastly, certain egg-laying substrates are observed in very few species. For example, Titanocheta *Scaptomyza* species are spider-egg breeders, and this trait appears to have evolved only once [19, 55]. Despite these challenges to taxon sampling, however, our analysis rejected the null model of Brownian evolution, and supported the hypothesis that ovariole number evolves in response to changes in egg-laying substrate across Hawaiian *Drosophila*.

The effect of larval substrate on fecundity in lab-reared and wild Hawaiian *Drosophila*

While species-specific ovariole numbers clearly have a strong heritable component even in the absence of the native substrate (Figure S1), the substrate does appear to provide chemical cues that are important to drive egg-laying behavior. In other words, substrate components may contribute to reproductive output by inducing or facilitating oviposition, but not by determining ovariole number. We therefore speculate that evolution of host specialization may have resulted from or in changes in heritable mechanisms that also, perhaps due to pleiotropy, determine ovariole number.

We note that other species of flies have also evolved differences in ovariole number when they shift between host substrates. For example, in African drosophilids and tephritid *Dacus* flies, generalist species that oviposit on a variety of egg-laying substrates have higher fecundity than specialists [9, 13, 29]. Moreover, specialist species of African and Central American *Drosophila* species are more fit in the presence of host-specific compounds [14, 56–58], some of which are toxic to other species of *Drosophila*. For example, *D. sechellia* is best reared on lab media supplemented with *Morinda* fruit [14], while *D. pachea* cannot be reared in laboratory conditions without supplementing media with sterols from its host cactus [59]. Egg-laying substrates for Hawaiian *Drosophila* have divergent chemical cues and fungal populations [60]. Laboratory populations of Hawaiian *Drosophila* often require extracts or pieces of egg laying substrates to stimulate oviposition, but can undergo development completely on laboratory food to give rise to adults with similar ovariole numbers as wild-caught flies (see STAR Methods; Figure S1). We therefore speculate that specific substrate components may not only allow females to distinguish between hosts for oviposition, but also may contribute to species- and substrate-specific egg laying behavior in Hawaiian *Drosophila*.

The effect of larval substrate on body and egg size

In addition to ovariole number, we tested whether shifts in larval ecology influenced the evolution of body size and egg index (calculated as the phylogenetic residual of egg volume to thorax volume), as these traits are often correlated with ovariole number and have been predicted to evolve in response to changes in ecology and reproductive strategy. For body size, we found that models that accounted for ecological evolution did not fit the data better than a Brownian Motion model (BM, $\Delta AICc > 2$). For egg index, models that accounted for ecological evolution fit the data better than BM and OU1 ($\Delta AICc > 2$), but we were unable to distinguish within between models ($\Delta AICc < 2$) (Table S6). These results suggest that the evolution of ovariole number, but not overall body size, has been linked to changes in larval ecology within the Hawaiian radiation of *Drosophila*.

The relationship between ovariole number and egg size

One of the life history characteristics commonly observed in animals is the inverse relationship between high reproductive capacity and investment into offspring [7]. Egg size is often considered a proxy for maternal investment in insects, and life history theory predicts a trade-off between maternal investment egg size and reproductive output, thereby predicting a negative correlation between ovariole number and egg size [61]. Previous empirical studies have found evidence for this predicted inverse correlation between egg size and ovariole number in some insects [14, 36].

We compared the evolutionary relationship between ovariole number and egg size across Hawaiian *Drosophila*, accounting for the relationship to body size in each variable using phylogenetic residuals [62]. We observed a significant negative correlation that explained most of the variation in relative egg size (Figures 3C and 3D; Data S1). Specifically, we found that when controlling for body size, species with more ovarioles have proportionally smaller eggs (Figures 3C and 3D; Data S1). This result suggests that the allometric relationship between ovariole number and egg size is complex, and implies that there are constraints preventing the evolution of both large eggs and high ovariole number.

METHOD DETAILS

Measurement of adult phenotypes

Adult ovaries were dissected in 1X PBS, and placed in 2% paraformaldehyde in 1X PBS overnight at 4°C. Ovaries were then stained with the nuclear dye Hoechst 33342 (Sigma, 1:500 of 10mg/ml stock solution) in 1X PBS for two hours at room temperature, then washed with 1X PBS for a total of one hour. Ovaries were mounted on glass slides in Vectashield mounting medium (Vector Labs), and ovarioles were spread apart using tungsten needles for species with high ovariole number. Adult ovariole number was counted from both ovaries to obtain ovariole number per female under fluorescent and white light microscopy using a Zeiss AxioImager microscope. Images of eggs were taken from these slides using DIC white light settings. Egg volume was calculated when mature eggs were laid by captive adult flies or when present in dissected ovaries. In these cases, egg volume was estimated by measuring the straight lines across the longest and widest points of the egg, and assuming a prolate spheroid shape following a previously published protocol [63] using ImageJ.

Adult bodies were placed in 99% ethanol after dissection for DNA extraction and adult size analysis. Lateral view images of the thorax were captured using a Zeiss Lumar Stereomicroscope. The highest point of the anterior tip of the thorax and the posterior-most point of the scutellum in the same image plane were used to measure thorax length. A straight line was drawn between these two points in these images using ImageJ's measure function. Thorax volume was calculated as thorax length $(mm)^3$ as a proxy for body size, and proportional egg volume was calculated by dividing egg volume by thorax volume. Raw data measurements for adult traits are publicly available at https://github.com/shchurch/hawaiian_Drosophila_ovaries_2018.

Measurement of larval phenotypes

Wandering larvae or early pupal stage individuals were dissected in 1X PBS + 0.1% Triton-X and fixed in 4% Paraformaldehyde in 1X PBS for 20 minutes at room temperature. Larval ovaries were stained as previously described [32] using mouse anti-Engrailed/Invected (4D9, Developmental Studies Hybridoma Bank, 1:50), FITC-conjugated Phalloidin (Sigma, 1:120), and Hoechst 33342 (Sigma, 1:500 of 10mg/ml stock solution). Samples were post-fixed in 4% paraformaldehyde in 1X PBS for 15 minutes at room temperature and mounted in Vectashield mounting medium (Vector Labs) for imaging using a Zeiss LSM780 Confocal Microscope at the Harvard Biological Imaging Center. Quantification of TFCs and TFs was conducted as previously described [32]. Larval ovary TF and TFC number measurements were obtained per ovary, unlike adult measurements, which were collected from both ovaries for each female.

For some samples, fewer collected specimens could be used for measuring total TF number than for others, as total TF number can only be counted in larval ovaries where TF morphogenesis has completed, which is usually near the end of larval development (2). At the time of larval ovary dissection, some ovaries contained completed TFs, while others were still undergoing morphogenesis and could not be used to gather data on TF number. In the latter cases, TFC number per TF was measured for those TFs that had completed morphogenesis, and total TFC number for that species was assigned based on the average TF number from other specimens from the same species. Larval trait raw data measurements are publicly available at https://github.com/shchurch/hawaiian_Drosophila_ovaries_2018.

Notes on analysis of larval ovarian development in Hawaiian *Drosophila*

Overall larval ovary morphology of Hawaiian *Drosophila* was similar to that of the *melanogaster* subgroup species (Figures 4D–4F), with characteristic TFC stacks forming toward the end of larval development (white arrowhead, Figures 4D–4F). To determine whether, as in *D. melanogaster* [32, 33, 38], ovariole number is established by the end of larval development and does not change during the pupal phase, we compared total TF number in Hawaiian *Drosophila* ovaries that had completed TF morphogenesis to adult ovariole number per ovary. We found a close to 1:1 correlation between TF number per ovary and ovariole number per ovary (Table S5).

One notable difference was in *S. caliginosa*, which had one ovariole per ovary in our adult samples, and two TFs per ovary in the developmental analysis. Given that Kambysellis and Heed [2] previously reported *S. caliginosa* females with more than two ovarioles, our result may be due to the small sample size of adults of *S. caliginosa* in our study (n = 5 versus n = 24 in the previous study), or to the difficulty of counting ovarioles in this species. However, we note that honeybees can destroy ovarioles that are formed during larval stages through programmed cell death during pupal development [64]. Thus, we cannot exclude the possibility that the difference between larval TF number (two) and adult ovariole number (one) observed in *S. caliginosa* may be a result of a similar developmental process as that reported in honeybees.

PCR amplification of mitochondrial genes for species identification

While there are detailed dichotomous keys for species identification of Hawaiian *Drosophila* [65], these keys focus on male-specific traits including male genitalia and other sexually dimorphic characters. Therefore, we identified female flies using a combination of morphological features [22, 66], collection site information, and DNA barcode-based methods as previously described [67]. Following ovary dissection, abdominal at tissue was used for DNA extraction using the QIAGEN Blood and Tissue kit. PCRs were conducted using primer sets (from 5′ to 3′) as previously published and listed in the KRT [18].

PCR was conducted using Dynazyme DNA polymerase (Thermo Scientific) as follows: 95°C 5 minutes, (95°C 30 s, 50°C (COI and COII) or 54°C (16S) 30 s, 72°C 30 s) x 30, 72°C 5 minutes. PCR products were cleaned using ExoSAP-IT (Affymetrix) and sequenced by Genewiz (Cambridge, MA). Sequences were analyzed using 4Peaks (Nucleobytes).

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of mitochondrial sequences for species delineation

All sequences were first analyzed by BLASTn alignment against the NCBI Nr/Nt collection, and the accession numbers were noted for those where there was one clear hit with 98%–100% sequence identity to one species (Table S2). Species that returned multiple 99%–100% BLASTn hits are summarized in Table S3. We note that in many cases, multiple hits are within a closely related species subgroup (Table S3), as reported in previous phylogenetic studies of Hawaiian *Drosophila* [18–21, 23, 68]. When BLAST results of all three barcodes were consistent, the sample was assigned a species identity. In cases where the BLAST analysis did not provide a clear identity, we tested whether the sample was sister to a single reference species in gene trees constructed using RAxML, as described below in 'Phylogenetic Inference'. Samples with unambiguous BLAST and/or tree-based support were assigned a species identity. Some samples resulted in BLAST and tree-based support for a closely related species group; in these cases, a species group identity was assigned instead of a specific species. Samples that did not have clear support for any species group hit were discarded from the dataset. The published and original sequence data, resultant gene trees, and custom scripts used for this analysis are available at https://github.com/shchurch/hawaiian_Drosophila_ovaries_2018, commit 5c70803, directory 'sequence_data_methods'.

Taxon sampling in this and previous studies

All measurements were taken following methods from [2], and 15 species from that study were also included in our study. The majority of the overlapping data were within two standard deviations of the previous study (Table S1; > 3 standard deviations highlighted in yellow), suggesting that these traits have remained stable over the last 40 years, such that our measurement methods are comparable to those of the previous study. We therefore included the data from Kambysellis and Heed [2] for subsequent analyses. Our final dataset for analysis contains 35 newly characterized species, 15 species included in both our field-caught dataset and Kambysellis and Heed [2], and 16 additional species discussed by Kambysellis and Heed [2] but not found by us in the field, yielding a total of 66 Hawaiian *Drosophila* species across all major species groups that were used in the analysis herein (Figure 1).

Our taxon sampling for phylogenetic inference combines the efforts of four previous studies [18, 19, 21, 23] and additional newly identified mitochondrial sequences (GenBank accession numbers MK276992 - MK277193). This sampling includes members of all major lineages of Hawaiian drosophilids. Nucleotide sequences from each of these four studies were downloaded from GenBank, totalling 18 genes. The sequence IDs were parsed using the program phyutility v2.2.6 [41], and the 18 genes were aligned individually using MAFFT v7.130b [42] with the "auto" option selected, and trimmed with Gblocks v0.91b [43] with the "with half" option selection. Trimmed sequences were concatenated using phyutility into two alignments, one including all 18 available genes and one including only the four mitochondrial genes. This second alignment reflects the analysis performed by O'Grady and colleagues [18]. PartitionFinder v1.1.1_Mac [44]; options 'raxml') was used to find the best fitting model for each partition; GTR + Γ +I was found for nearly all partitions. For species delineations, sequences of the three targeted genes generated in this study were combined with homologous sequences from the four previous studies and aligned and trimmed using the same procedure as above. Gene trees were generated in RAxML v8.2.3 [45] using a GTR + Γ +I model of sequence evolution).

Phylogenetic inference

Phylogenetic relationships and divergence time estimates were inferred simultaneously using both the mitochondrial and mitochondrial+nuclear alignments in a Bayesian framework in BEAST v. 2.3.2 [46, 47]. A single calibration at the root (i.e., at the base of Hawaiian *Drosophila* + *Scaptomyza*) was used to infer divergence times; this was assigned a uniform prior from 23.9–37.1 Ma, following [23]. Rate-smoothing was performed using a relaxed lognormal clock model [69]. The BEAST analyses followed the same partitioning scheme as RAxML, as determined by PartitionFinder [44] and utilized a birth-death tree prior. Four separate chains were allowed to run for 100 million generations (sampling every 10,000) using the CIPRES supercomputer cluster (https://www.phylo.org). Convergence was assessed using the AWTY web interface [48] and effective sample size (ESS) values of the runs (using values > 200 as a cutoff) in Tracer [49]. After convergence was reached, the individual runs were combined and the maximum clade credibility tree, including credibility intervals (CI) for ages and posterior probabilities (PP) for node support, was assembled in TreeAnnotator [50]. Upstream phylogenetic comparative analyses used either the maximum clade credibility (MCC) tree or a subset of 500 trees from the posterior distribution of trees, as appropriate; analyses were repeated for each of the two BEAST analyses. The final alignments, mcc trees, and posterior distributions are available at https://github.com/shchurch/hawaiian_Drosophila_ovaries_2018, commit 5c70803, directory 'sequence_data_methods'.

Phylogenetic relationships between Hawaiian *Drosophila* in this study

Our study focuses on the Hawaiian clade Drosophilidae, which comprises an estimated 1000 species in two genera, *Drosophila* and *Scaptomyza*. The phylogeny of this clade and its substituent subclades has been the focus of many recent studies [18–21, 23, 68]. The monophyly of Hawaiian drosophilids is well-supported, as is the monophyly of *Scaptomyza* and Hawaiian *Drosophila* within

them. The relationships between and within the subgroups of Hawaiian *Drosophila* (*Scaptomyza*, PW, MM, Haleakala, and AMC species groups) remain less resolved. Specifically, the monophyly of each of these individual groups is generally well-supported (but see the moderate support values for the MM group reported in [21] and [18]), but the relationships between these large groups are in conflict across studies.

To generate a phylogenetic tree for evolutionary modeling analysis, we compiled data from published phylogenetic studies and captured the two topologies of Hawaiian *Drosophila* species groups that have been published to date. The consensus tree topology that we recovered matched the topology recovered in recently published phylogenetic analyses [18, 21], and the second most common basal topology matched the alternative topology presented in [21]. Our phylogenetic analysis also captured monophyletic species subgroups in AMC, PW and *Scaptomyza* [19, 21, 23]. Given that species relationships at the species group and subgroup level were recovered in the phylogenies used for phylogenetic comparative methods analysis, we believe our analysis represents an accurate estimate of our current knowledge of Hawaiian *Drosophila* phylogeny.

Phylogenetic Generalized Least-squares Analyses

All phylogenetic comparative analyses and corresponding figures were computed in R version 3.2.0 [51]. The evolutionary relationship between ovariole number, egg volume, egg volume over thorax volume, and thorax volume was analyzed in pairs using phylogenetic generalized least-squares in **nlme** v.3.1-121 [28], using the phylogenetic correlation matrix generated using the *corMartins* function in **ape** v.3.3 [52, 53] with a small starting alpha value. All traits were natural log transformed prior to analysis. Pairwise comparisons were performed over 100 trees randomly drawn from the posterior distribution generated in BEAST. The range and average of both the p value and the slope from the PGLS models across the 100 trees were calculated, and a cutoff threshold of 0.05 was used to determine significance of the p values.

Analysis of Evolutionary Regimes

We used all reported ecological information about Hawaiian *Drosophila* as summarized by Magnacca and colleagues [16] to code oviposition site for the species in our dataset. Three different coding schemes were compared: (1) OU8, which considered eight ecological substrates (bark, flower, fruit, fungus, generalist, leaf, sap and spider egg breeders); (2) OU3, which considered three states (bark, flower & spider egg, and 'other substrate' breeders); and (3) a final one with two states (OU2: bark and non-bark breeders). We categorized species as bark breeders if they utilized the tree stem or trunk, though previous studies distinguished between the two [3, 4]. Ancestral states for each of these character codings were calculated over 100 trees randomly drawn from the posterior distribution of trees generated with BEAST. The most likely ecological state was mapped at each node using the *rayDISC* function in the R package **corHMM**, v.1.18 [25], and the resulting tree was pruned to include only tips with ovariole number data.

The fit of three models of trait evolution were assessed on pruned trees using the R package **OUwie** v.1.48 [25]. The three models tested were Brownian Motion (BM1), Ornstein-Uhlenbeck with a single optimum for all species (OU1), and Ornstein-Uhlenbeck with optima for each ecological state (OUM; OUM was fit for eight, three and two state models for each of the distinct character codings, respectively OU8, OU3, and OU2 as described above). Corrected Akaike Information Criterion (AICc) values were compared for each of these models for each of the trait coding schemes, where the best-fit model (i.e., the model with the lowest delta AICc score) was moderately supported when other models had delta AICc of 2-10, and strongly supported when delta AICc > 10. This analysis was repeated over each of the 100 trees, and the frequency of each best-fitting model was recorded. Optimized theta values for each of the three OUM analyses were untransformed and recorded (Data S1).

DATA AND SOFTWARE AVAILABILITY

All of the commands and data used to perform phylogenetic comparative analyses, as well as the corresponding commands to generate the figures, are available in the public repository https://github.com/shchurch/hawaiian_Drosophila_ovaries_2018, commit 5c70803.

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Supplemental Information

Reproductive Capacity Evolves in Response to Ecology through Common Changes in Cell Number in Hawaiian *Drosophila*

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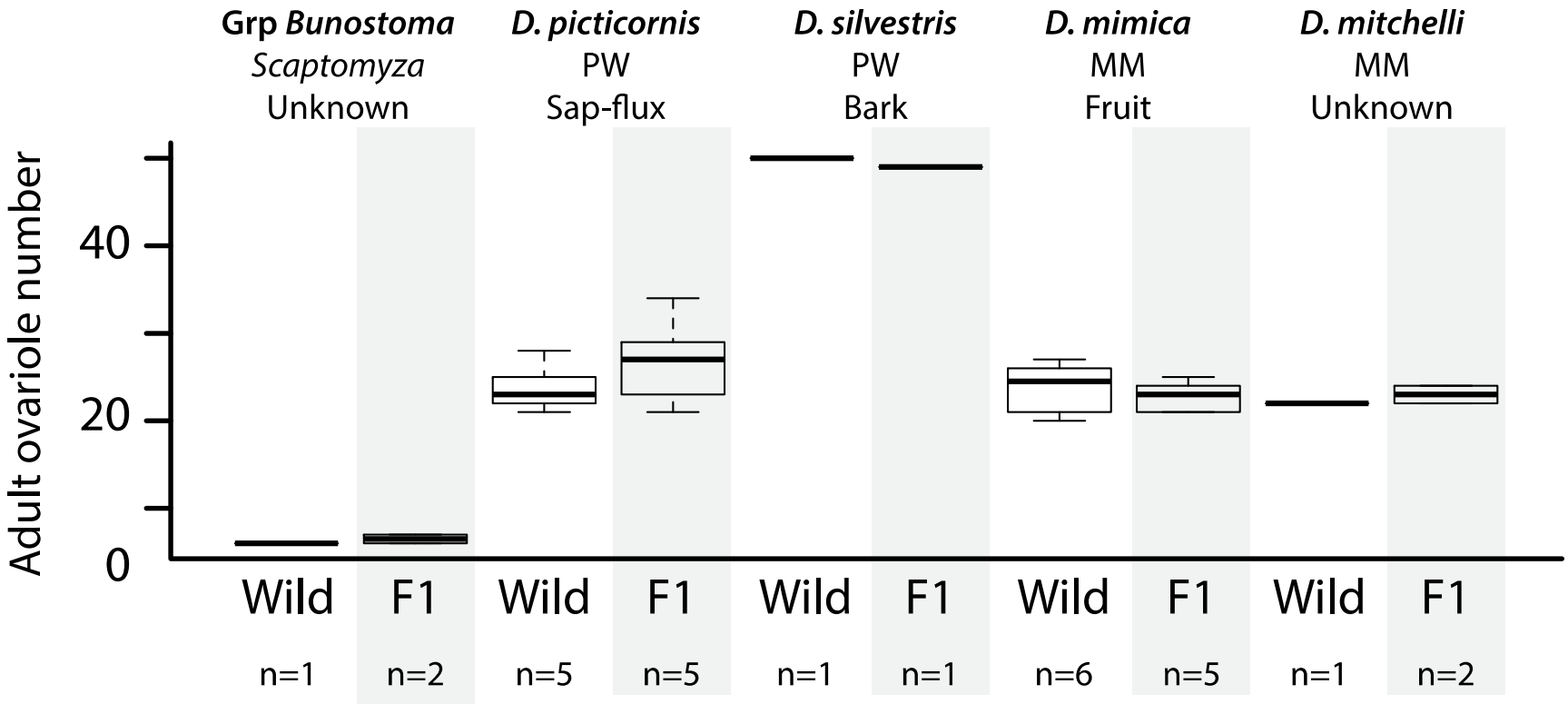

Figure S1. Comparison of mean ovariole number of wild caught females and their F1 offspring reared in the laboratory; Related to Figure 1. Box plot of ovariole number from wild-caught females and their F1 offspring reared in the same laboratory condition for each species. Species name is indicated along with the species group (*Scaptomyza*, PW for picture wing and MM for modified mouthpart) and oviposition substrate. Ovariole number is not significantly different between wild-caught females and F1 females for any species, regardless of oviposition substrate, natural or laboratory diet. Sample size (number of adults) is indicated below the plot.

PART 1		All species groups			Picture wing spp.			AMC spp.			Mod. mouthpart spp.			Scaptomyza spp.		
		min	avg	max	min	avg	max	min	avg	max	min	avg	max	min	avg	max
ON - Thorax volume (mm3)	Slope	0.234	0.292	0.500	0.412	0.416	0.424	0.014	0.019	0.020	0.572	0.598	0.627	-0.307	-0.284	-0.276
	p-value	**0.000**	**0.002**	0.011	**0.000**	**0.000**	**0.000**	0.841	0.845	0.892	**0.001**	**0.004**	**0.008**	0.134	0.150	0.174
Egg vol (um3) - Thorax volume (mm3)	Slope	0.156	0.353	0.407	0.164	0.185	0.164	0.745	0.748	0.760	-0.038	-0.038	-0.037	0.654	0.679	0.680
	p-value	**0.000**	**0.000**	0.058	0.086	0.109	0.164	**0.000**	**0.000**	**0.000**	0.811	0.811	0.811	**0.012**	**0.012**	**0.016**
ON - Proportional Egg volume (um3/mm3)	Slope	-0.649	-0.570	-0.532	-0.453	-0.445	-0.438	-0.321	-0.321	-0.314	-0.686	-0.659	-0.648	-0.570	-0.473	-0.367
	p-value	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.007**	**0.008**	**0.010**	**0.000**	**0.001**	**0.001**	0.084	0.170	0.306
ON - Egg volume (um3)	Slope	-0.703	-0.42	-0.376	-0.088	-0.081	-0.07	-0.308	-0.222	-0.161	-0.69	-0.689	-0.689	-0.784	-0.676	-0.567
	p-value	**0.000**	**0.000**	**0.000**	0.674	0.695	0.739	**0.008**	0.049	0.127	0.396	0.396	0.397	**0.001**	**0.003**	**0.007**

PART 2		nc + mtDNA						mtDNA					
		Combined data			Present data			Combined data			Present data		
		min	avg	max	min	avg	max	min	avg	max	min	avg	max
ON - Thorax volume (mm3)	Slope	0.218	0.274	0.371	0.22	0.291	0.393	0.218	0.274	0.371	0.257	0.321	0.388
	p-value	**<0.000**	**0.004**	**0.017**	**<0.000**	**0.01**	**0.04**	**<0.000**	**0.004**	0.017	**<0.000**	**0.004**	0.017
Egg vol (um3) - Thorax volume (mm3)	Slope	0.285	0.371	0.426	0.153	0.201	0.255	0.285	0.371	0.426	0.124	0.199	0.276
	p-value	**<0.000**	**<0.000**	**0.003**	0.021	0.054	0.102	**<0.000**	**<0.000**	**0.003**	0.017	0.072	0.212
ON - Egg/Thorax volume	Slope	-0.612	-0.583	-0.554	-0.566	-0.564	-0.562	-0.611	-0.583	-0.554	-0.57	-0.57	-0.568
	p-value	**<0.000**	**<0.000**	**<0.000**	**<0.000**	**<0.000**	**<0.000**	**<0.000**	**<0.000**	**<0.000**	**<0.000**	**<0.000**	**<0.000**
ON - Egg volume	Slope	-0.418	-0.383	-0.344	-0.528	-0.48	-0.427	-0.418	-0.383	-0.344	-0.51	-0.425	-0.349
	p-value	**<0.000**	**<0.000**	**0.001**	**<0.000**	**<0.000**	**0.001**	**<0.000**	**<0.000**	**0.001**	**<0.000**	**0.002**	**0.01**

Table S4. Summary of Phylogenetic Generalized Least Squares (PGLS) analyses. Related to Figure 3. (Part 1) PGLS analyses of adult reproductive traits in Hawaiian *Drosophila*. PGLS analysis of relationships between ovariole number and thorax volume (mm^3), egg volume (μm^3) and thorax volume, and ovariole number and proportional egg volume ($\mu m^3/mm^3$) are listed. Regression analyses were performed with the R package **nlme** v.3.1-121 [S1] on 100 trees from a BEAST posterior distribution using nuclear and mitochondrial genes, and the minimum, average, and maximum slope and p-value for the analysis is included in the table. P-values below 0.01 are in bold.**(Part 2)** PGLS analyses on nuclear and mitochondrial or mitochondrial gene only BEAST trees. Relationships between ovariole number and thorax volume, ovariole number (ON) and egg volume, egg volume and thorax volume, and ovariole number and thorax/egg proportion are listed for regression analyses that were conducted across 100 trees from a BEAST posterior distribution using nuclear and mitochondrial genes or mitochondrial genes only. “Combined data” includes present data and Kambysellis and Heed [S2]. “Present data” indicates analyses conducted using data collected from this study alone. Minimum, average, and maximum slope and p-value for the analysis is included in the table. P-values below 0.01 are indicated in bold.

Species ID	Spp. group	Substrate	TF #	sd	n	Adult ON per ovary	n	TF:ON Ratio	TFC # per TF	sd	Total TFC #	sd	n
D. basimacula	AMC	Leaf	7.00 ±	0.53	8	6.24	21	1.12	8.96 ±	1.19	62.59 ±	8.41	8
D. tanythrix	AMC	Leaf	5.75 ±	0.96	4	5.79	7	0.99	10.55 ±	0.86	61.03 ±	13.65	3
D. mimica	MM	Fruit	11.00 ±	1.41	2	11.62	13	0.95	7.50 ±	0.14	106.90 ±	43.70	2
D. mitchelli	MM	Unknown	11.50 ±	0.71	2	11.20	3	1.03	7.80 ±	0.57	89.50 ±	0.99	2
D. grimshawi	PW	Bark	25.25 ±	3.96	5	23.90	8	1.06	9.58 ±	1.39	193.47 ±	57.89	8
D. hawaiiensis	PW	Sap flux	17.67 ±	2.08	3	18.64	7	0.95	9.47 ±	1.04	167.97 ±	33.86	3
D. picticornis	PW	Sap flux	13.00 ±	2.83	2	12.63	12	1.03	11.18 ±	1.23	144.84 ±	17.48	9
D. setosimentum	PW	Bark	19.00 ±	2.82	2	20.60	4	0.92	9.87 ±	0.15	186.90 ±	20.63	3
D. silvestris	PW	Bark	18.00 ±	0.00	1	24.50	2	0.73	12.00 ±	2.68	216.00 ±	48.37	2
D. villocipedis	PW	Bark	29.00 ±	1.26	4	20.70	5	1.40	10.02 ±	0.43	218.01 ±	14.92	6
Bunostoma spp	Scaptomyza	Unknown	6.00 ±	0.00	2	6.50	10	0.92	9.40 ±	1.56	56.50 ±	9.19	2
S. caliginosa	Scaptomyza	Flower	2.00 ±	0.00	2	1.00	5	2.00	7.75 ±	0.35	15.50 ±	0.71	2
						Average TF:ON F		1.092					

Table S5. Summary of TF number, TFC number per TF, and total TFC number from Hawaiian *Drosophila* larval ovaries; Related to Figure 4. TF number and total TFC number are per larval ovary**,** comparison of TF number observed per developing ovary compared to average ovariole number (ON) per ovary, and the ratio of TF number to ovariole number for various species. Sample size (n) and standard deviation (sd) is indicated for each species.

		min	avg	max
TF # - TFC # per TF	Slope	0.320	0.744	1.728
	p-value	0.199	0.376	0.647
TF # - Total TFC #	Slope	0.873	0.873	0.873
	p-value	**0.000**	**0.000**	**0.000**
TFC # per TF - Total TFC #	Slope	0.097	0.097	0.097
	p-value	0.059	0.059	0.059

Table S6. Phylogenetic Generalized Least Squares (PGLS) analysis of larval ovarian measurements in Hawaiian *Drosophila*; Related to Figure 4. Relationships between TF number and TFC number per TF, TF number and total TFC number, and total TFC number and TFC number per TF are listed. Regression analyses were performed with the R package **nlme** v.3.1-121 [S1] on 100 trees from a BEAST posterior distribution using nuclear and mitochondrial genes, and the minimum, average, and maximum slope and p-value for the analysis is included in the table. P-values below 0.01 are indicated in bold.

Supplemental References

S1. Beaulieu, J.M., Oliver, J.C., and O'Meara, B. (2012). corHMM: Hidden Markov Models in R, Version 1.0.

S2. Kambysellis, M.P., and Heed, W.B. (1971). Studies of Oogenesis in Natural Populations of Drosophilidae. I. Relation of ovarian development and ecological habitats of the hawaiian species. Am. Nat. 941, 31-49.

S3. Beaulieu, J.M., and O'Meara, B. (2014). OUwie: Analysis of Evolutionary Rates in an OU Framework. R package version 1.