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1 **Reproductive capacity evolves in response to ecology through common developmental**
2 **mechanisms in Hawai'ian *Drosophila***

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26 *Short Title*: Reproductive evolution through ecology and development

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30 **Abstract**

31 Lifetime reproductive capacity, or the total number of offspring that an individual can give rise
32 to in its lifetime, is a fitness component critical to the evolutionary process. In insects, female
33 reproductive capacity is largely determined by the number of ovarioles, the egg-producing
34 subunits of the ovary. Recent work has provided insights into the genetic and environmental
35 control of ovariole number in *Drosophila melanogaster*. However, whether regulatory
36 mechanisms discovered under laboratory conditions also explain evolutionary variation in
37 natural populations is an outstanding question. Here we report, for the first time, insights into the
38 mechanisms regulating ovariole number and its evolution among Hawai'ian *Drosophila*, a large
39 adaptive radiation of fruit flies in which the highest and lowest ovariole numbers of the genus
40 have evolved within 25 million years. Using phylogenetic comparative methods, we show that
41 ovariole number variation among Hawai'ian *Drosophila* is best explained by adaptation to
42 specific oviposition substrates. Further, we show that evolution of oviposition on ephemeral egg-
43 laying substrates is linked to changes the allometric relationship between body size and ovariole
44 number. Finally, we provide evidence that the developmental mechanism principally responsible
45 for controlling ovariole number in *D. melanogaster* also regulates ovariole number in natural
46 populations of Hawai'ian drosophilids. By integrating ecology, organismal growth, and cell
47 behavior during development to understand the evolution of ovariole number, this work connects
48 the ultimate and proximate mechanisms of evolutionary change in reproductive capacity.

49

50 **Keywords:** ovary, ovariole, terminal filament, adaptive radiation, allometry, constraint

51 **Introduction**

52 Reproductive capacity is an important life history trait that directly influences fitness by
53 determining how many offspring an individual can leave behind. There is a wide range in
54 potential fecundity across species (1, 2), which is often interpreted as trade-offs with presumed
55 ecological and developmental constraints . Trade-offs have been invoked to explain patterns of
56 egg-laying in animals, where total fecundity can correlate negatively with egg mass, clutch size
57 or lifespan (3-10), and positively with body size (11-13). In addition to these hypothesized
58 physical or growth-related constraints, life history parameters including predation risk,
59 environmental variability, host specialization and levels of parental care have been proposed to
60 influence evolutionary change in fecundity (1, 14-17), suggesting that this trait could represent a
61 complex intersection between ecology and physiology. However, few studies have addressed
62 how female reproductive capacity evolves in response to ecology, and how these pressures
63 manifest as different phenotypes through changes in development.

64 In insects, female reproductive capacity is strongly influenced by the number of egg-
65 producing structures called ovarioles (1, 18-23). Ovariole number is species-specific and
66 genetically determined (24, 25). Most insects have limited intraspecific variation in ovariole
67 number, and the effect of ovariole number on fecundity has been observed by comparing mean
68 ovariole numbers within or between species. In many insects, including beetles, fruit flies, and
69 aphids, ovariole number is positively correlated with fecundity between and within species (1,
70 21-23). For example, *Drosophila melanogaster* strains with naturally occurring or genetically
71 manipulated higher ovariole numbers both show increased fecundity (18, 26). While
72 physiological traits like egg production rate may also play an important role in determining
73 reproductive capacity (27), these can be difficult to assess in laboratory settings where egg-

74 laying conditions may not be suitable for some insects. In contrast, ovariole number has served
75 as a proxy for reproductive capacity for decades (18), as it is a quantitative trait that can be easily
76 measured from field and laboratory samples.

77 Ovariole number is established during larval and pupal stages (20), and can be affected
78 by environmental conditions during this phase of development, including nutrition and
79 temperature (24, 28, 29). During larval development, a specific group of cells called terminal
80 filament cells (TFCs) form stacks called terminal filaments (TFs) that serve as the beginning
81 point of each ovariole (30-33). Developmental mechanisms of ovariole number evolution are
82 best characterized in species of the African *melanogaster* subgroup of *Drosophila*, where
83 average ovariole number ranges from 43 to 18 per female (1, 34), and ovariole number
84 differences result primarily from changes in TFC number (29, 35). Ovariole number is highly
85 polygenic and regulated by pleiotropic genes (25), thus offering an opportunity to study the
86 evolution of a complex quantitative trait in response to different environments.

87 Major shifts in ovariole number have been attributed to aspects of life history.
88 Ovoviviparity, where females oviposit first instar larvae, is often correlated with reduced
89 ovariole number (16), suggesting that increased parental investment is linked to reduced
90 fecundity as observed in other animals (17). The stability of the environment and the
91 predictability of egg-laying substrates may influence evolution of ovariole number, as more
92 stable environments or abundant substrates are correlated with higher ovariole number, and
93 species occupying unpredictable environments or scarce substrates tend to have lower ovariole
94 numbers (15, 36). In the well-studied *Drosophila melanogaster* subgroup, previous studies have
95 suggested that reproductive strategies and ovariole number evolve in response to oviposition or
96 larval nutrition substrate (35-37). Most *melanogaster* subgroup species are generalists that

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97 oviposit on a variety of decaying fruits, and mean ovariole number in this subgroup ranges from
98 43 to 18 per female (1, 34). In contrast, *D. erecta* and *D. sechellia* are specialists on *Pandanus*
99 fruit and the toxic *Morinda* fruit, respectively (38, 39), and *D. sechellia* has the lowest reported
100 ovariole number of the group (1). This reduction in ovariole number has been hypothesized to be
101 the result of increased egg size as an adaptation to feeding on the toxic *Morinda* (40), or to be
102 due to lower insulin signaling levels evolved in response to the relatively constant nutritional
103 input provided by substrate specialization (35). Reviewing data on oviposition behavior in
104 *melanogaster* subgroup species, Lachaise (37) proposed that the high ovariole number observed
105 in the generalists *D. melanogaster* and *D. simulans* may be driven by the frequent oviposition
106 opportunities available to these species, as they oviposit on most decaying fruit. However, the
107 *melanogaster* subgroup is not well-suited for a broader understanding of ovariole number
108 evolution, as most species share similar oviposition substrates (i.e. rotting fruit) and there are few
109 independent instances of evolution of specialists.

110 In contrast, Hawai'ian *Drosophila* have evolved to specialize on a variety of oviposition
111 substrates, including decaying flowers, leaves, fungi, sap fluxes, and bark of native plants, and
112 eggs of native spiders (41). Moreover, these flies exhibit the most extreme interspecies range of
113 ovariole number reported in the genus, ranging from two to 101 per ovary (42). Hawai'ian
114 *Drosophila* have undergone rapid island radiation from a common ancestor in the last 25 million
115 years, leading to over 1000 extant species (43-45). The high species diversity of Hawai'ian
116 *Drosophila* is spread across five monophyletic species groups that share genetic, morphological
117 and ecological similarities, and rely on different oviposition substrates (44, 46-48), as follows
118 (Figure 1): *Scaptomyza* are small species that primarily lay eggs on leaves or flowers. Picture
119 wing (PW) species are larger species with striking pigment patterns on their wings (49). PW

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120 species primarily lay eggs on decaying bark or branches of native trees, though some specialize
121 on sap fluxes (41). Modified mouthpart (MM) species, which have male-specific modifications
122 on mouthparts used during mating (50), have the largest range of egg-laying substrates,
123 specializing on decaying leaves, fungi, sap or bark (51). Haleakala species are darkly pigmented
124 flies that only lay eggs on native fungi. Lastly, most antopocerus-modified tarsus-ciliated tarsus
125 (AMC) species are leaf breeders, though there are a few exceptions that have evolved bark-
126 breeding (44).

127 Ovariole number is highest in the PW species (up to 202 per female), and lowest in
128 *Scaptomyza* and AMC species (as few as 2 per female) (42). Dramatic differences in ovariole
129 number between species have been hypothesized to be a result of shifts between their varied
130 oviposition substrates (42, 51). Other studies have posited that the divergent ovariole number
131 observed in Hawai'ian *Drosophila* may be a result of r-K evolution (42), given the surface area
132 of decaying trees, and the predictability of this substrate in the field (36), is greater than that of
133 other oviposition substrates (51, 52). However, the studies supporting these hypotheses primarily
134 sampled PW species, and used phylogenies that have since been substantially improved upon in
135 more recent studies that include expanded taxon sampling and additional loci (44, 46, 48, 53).

136 To investigate the linked effects of ecology and development underlying ovariole number
137 evolution in Hawai'ian *Drosophila*, we conducted phylogenetic comparative analyses of life
138 history traits from 60 species, and comparative development analyses from ten species using
139 both wild-caught flies and laboratory strains. Our results identify potential mechanisms of
140 evolutionary change in ovariole number operating at three levels of biological organization. First,
141 we found that evolutionary shifts in ecological niche could predict the dramatic differences in
142 ovariole number in Hawai'ian *Drosophila*. Second, whether adult body size was coupled with

143 ovariole number or egg volume differed between species groups with different oviposition
144 substrates, suggesting that the allometric growth relationships between these traits evolves
145 dynamically. Finally, we found that changes in ovariole number from two to 60 per individual
146 can be explained by changes in total TFC number, suggesting that ovariole number diversity
147 evolves through the same developmental mechanism, regardless of the specific ecological
148 constraints or selective pressures.

149

150 **Results and Discussion**

151 *Adult reproductive traits of Hawai'ian Drosophila*

152 We measured three major adult traits relevant to reproductive capacity (body size,
153 ovariole number and egg volume), from field-collected females, lab-reared F1 offspring of field-
154 collected females, and females from laboratory strains (Figure 1; Table S1). Species identities of
155 field-collected females were assigned based on morphological keys or DNA barcoding (Tables
156 S2, S3). All traits ranged over an order of magnitude within Hawai'ian *Drosophila*: body size
157 ranged from 0.71mm for *S. devexa* to 3.12mm for *D. melanocephala*, ovariole number per
158 female ranged from 2 for *S. caliginosa* to 88.5 for *D. melanocephala*, and egg volume ranged
159 from 0.01 μm^3 for *Bunostoma spp.* group (*S. palmae*/*S. anomala*) to 0.2 μm^3 for *D. adunca*,
160 highlighting the diversity of life history traits in Hawai'ian *Drosophila*.

161 Within the *melanogaster* subgroup species, species-specific differences in ovariole
162 number are largely heritable (25, 54, 55). To test whether this is also the case in Hawai'ian
163 *Drosophila*, we compared ovariole number of wild-caught females and their lab-reared F1
164 offspring, across five species with different egg-laying substrates. We observed no significant
165 differences between the ovariole numbers of these two generations regardless of natural substrate

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166 (Figure S1), indicating that species-specific differences in ovariole number are also strongly
167 genetically determined in Hawai'ian *Drosophila*.

168

169 *Larval ecology influences ovariole number evolution*

170 A previous study based almost exclusively on picture wing species proposed that
171 evolutionary shifts in larval ecology had driven ovariole number diversification in these flies
172 (51). To test this hypothesis across the major groups of Hawai'ian *Drosophila*, we compared the
173 fit of evolutionary models of ovariole number that accounted for ecologically driven evolution,
174 to those that did not. Our dataset included both specialist species that oviposit on one of bark, sap
175 flux, leaf, fungus, fruit, flower or spider-eggs, as well as generalist species that oviposit on
176 multiple decaying substrates (Figure S2). We compared the fit of five models to our data, two of
177 which ((i) Brownian Motion, BM, and (ii) an Ornstein Uhlenbeck model with a shared optimum
178 for all species, OU1) do not take into account the oviposition substrate, and three of which were
179 nested ecological models based on alternative methods of substrate classification: (iii) OU2
180 assumed two states, bark breeders and all other species, to test previous suggestions that bark-
181 breeding may drive evolution of ovariole number (51, 52); (iv) OU3 assumed three states,
182 *Scaptomyza* specialists on spider eggs and flowers, bark-breeders, and species using any other
183 substrate, to test proposals that substrates influence ovariole number evolution because of their
184 differences in carrying capacity and field predictability (36, 42); and (v) OU8 categorized each
185 oviposition substrate separately. These five models were fit over 100 trees sampled from the
186 posterior distribution of a Bayesian phylogenetic analysis to account for phylogenetic
187 uncertainty.

188 We found that models accounting for larval ecology explained the ovariole number
189 diversification in Hawai'ian *Drosophila* (Table 1) better than those that did not. Comparing the
190 three ecological models, we found that the three-state model (OU3), which accounted for both
191 bark breeders and *Scaptomyza* specialists, was supported as the best-fit model across a majority
192 of trees for ovariole number ($\Delta\text{AICc} > 2$ as compared to OU2 and OU8 models; Table S4).
193 Estimated theta values for the OU3 model showed that bark breeders have more ovarioles than
194 species that oviposit on other substrates, suggesting that evolution of higher ovariole numbers
195 accompanied the transition to bark breeding from likely non-bark breeding ancestors (Fig. 2A,B,
196 Table S5), consistent with earlier hypotheses (51, 52). In contrast, *Scaptomyza* species may have
197 experienced a dramatic decrease in ovariole number as they independently specialized on spider
198 eggs and flowers (Fig. 2B). Taken together, our results suggest that shifts in oviposition substrate
199 may have contributed to the evolution of diverse ovariole numbers in this group, not only for
200 picture wing flies as predicted previously (51), but across the adaptive radiation of Hawai'ian
201 *Drosophila*.

202 In African drosophilids and tephritid *Dacus* flies, generalist species that oviposit on a
203 variety of egg-laying substrates have higher fecundity than specialists (1, 22, 37). Moreover,
204 specialist species of African and Central American *Drosophila* species are more fit in the
205 presence of host-specific compounds (40, 56-58), some of which are toxic to other species of
206 *Drosophila*. For example, *D. sechellia* is best reared on lab media supplemented with *Morinda*
207 fruit (40), while *D. pachea* cannot be reared in laboratory conditions without supplementing
208 media with sterols from its host cactus (59). Egg-laying substrates for Hawai'ian *Drosophila*
209 have divergent chemical cues and fungal populations (60). Hawai'ian *Drosophila* often lay few
210 eggs on unsupplemented laboratory food (see Supplemental Information), but do not change

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211 ovariole number when reared on this food (Figure S1). We therefore speculate that specific
212 substrate components may not only allow females to distinguish between hosts for oviposition,
213 but also may contribute to species- and substrate-specific egg laying behavior in Hawai'ian
214 *Drosophila*.

215

216 *Evolution of specialist habitats changes allometry of reproductive traits*

217 The range of Hawai'ian *Drosophila* body sizes is greater than that of other members of
218 the genus, spanning an order of magnitude (Table S1). To determine whether changes in
219 allometric growth might underlie reproductive trait evolution, we analyzed the allometric ratio of
220 such traits using a phylogenetic least squares (PGLS) analysis and thorax volume (thorax
221 length³) as a proxy for body size. We found that across all Hawai'ian *Drosophila*, thorax volume
222 was significantly positively correlated with both ovariole number (Figure 3A; Table 2; Table S6)
223 and egg volume (Figure 3B; Table 2; Table S6). However, individual species groups show
224 differences in trends for allometric ratios of reproductive traits. In PW and MM species, body
225 size is correlated positively with ovariole number (Figure 3A1, A2), but not with egg volume
226 (Figure 3B1, B2). In contrast, AMC and *Scaptomyza* species have a positive correlation with
227 body size and egg volume (Figure 3B3, B4), but not ovariole number (Figure 3A3, A4). For PW,
228 MM, and AMC, there is a negative correlation between ovariole number and proportional egg
229 size (Table S2; Figure S3B-D), and there is a negative correlation between ovariole number and
230 egg volume in AMC and *Scaptomyza* (Table 2; Figure S3I-J).

231 We note that these trends are associated with differences in life history strategies between
232 groups. PW and MM group species, in which ovariole number increases with increasing body
233 size (Figure 3A1, A2), lay eggs in abundant and varied substrates (41): PW are primarily bark

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234 breeders that oviposit eggs in clutches of up to 100 eggs (42), and MM group species can occupy
235 a wide range of oviposition preferences, including bark, leaf, fruit, fungus, and sap flux (41). In
236 contrast, species of AMC and *Scaptomyza*, in which ovariole number and body size are
237 decoupled (Figure 3A3, A4), have independently evolved use of substrates with low carrying
238 capacity: AMC group species are primarily leaf breeders, reproducing on damp leaves in the
239 forest bed, while the oviposition substrates of *Scaptomyza* species include ephemeral substrates,
240 such as flowers, spider eggs and fresh leaves, many of which are not occupied by other
241 Hawai'ian *Drosophila* species groups (41). In sum, while a positive correlation between body
242 size and fecundity is commonly posited in egg-laying animals (11, 13), we did not find universal
243 support for this trend across Hawai'ian *Drosophila*. This is, however, consistent with previous
244 studies on Diptera, wherein trends toward higher fecundity or ovariole number in larger animals
245 observed within species (11) contrast with between-species differences in ovariole number that
246 do not always correlate with body size (22, 37, 61).

247

248 *Larval ovary somatic cell number determines ovariole number*

249 We previously identified two developmental mechanisms that can alter ovariole number
250 during development: changes in TFC number per TF and change in total TFC number (29). To
251 determine whether the same developmental mechanisms that regulate ovariole number in
252 laboratory populations, also underlie the evolution of ovariole number in natural populations, we
253 measured TF and TFC numbers in the developing larval ovaries of Hawai'ian *Drosophila*. Our
254 analysis of 12 species representing four of the major Hawai'ian *Drosophila* species groups
255 showed that even over a range of ovariole numbers spanning an order of magnitude (Figure 4;
256 Table S7), larval TF number essentially corresponded to adult ovariole number (Table S8).

257 Although TFC number per TF varied somewhat between species (Figure 4A; Table S7), PGLS
258 analysis showed no correlation between TFC number per TF and total TF number (Table 3). In
259 contrast, average total TFC number was strongly positively correlated with TF number (Table 3;
260 Figure 4B; Table S7), suggesting that, as in laboratory populations of *D. melanogaster*, changes
261 in TFC number underlie ovariole number evolution in Hawai'ian *Drosophila*.

262 The developmental mechanism underlying ovariole number evolution is particularly
263 interesting in light of the allometric changes in Hawai'ian *Drosophila* species groups. There has
264 been some debate as to whether allometry constrains or facilitates adaptive evolution (62-64). In
265 Hawai'ian *Drosophila*, the allometric relationship between two important female reproductive
266 traits, ovariole number and egg size, was coupled to body size in different groups in different
267 ways: when ovariole number was coupled with body size, egg size was not, and vice versa
268 (Figure 3). These trends were associated with abundant versus scarce egg-laying substrates
269 respectively (Figure 1). While the phenotypic integration of ovariole number and egg volume
270 appears tightly regulated across insects (65), the coupling of ovariole number to body size
271 appears more flexible in Hawai'ian *Drosophila*, suggesting that in this context, heritable changes
272 in allometry may contribute to adaptive evolution.

273 Ovariole number is regulated by both by intrinsic and extrinsic growth factors, including
274 Hippo signaling, ecdysone and insulin-like peptides, all of which can also regulate body size (26,
275 35, 66-68). Thus, we propose that the mechanistic basis for evolutionary change of ovariole
276 number on different substrates, may be changes in the relative influence of nutritionally
277 regulated circulating growth factors on the one hand, and cell-autonomous growth on the other
278 hand, on ovarian development during larval and pupal stages. For example, we speculate that on
279 certain substrates, the larval ovary may become less sensitive to nutritionally-mediated growth

280 factors by evolving lower expression levels of growth factor receptors, and relying more on
281 tissue-specific growth factors, which could include local insulin release or cell proliferation
282 pathways such as Hippo signaling.

283 Taken together, we found that highly divergent ovariole number, and by proxy female
284 reproductive capacity, have evolved together with changes in egg-laying substrate across
285 Hawai'ian *Drosophila*. Moreover, this remarkable adaptive radiation is linked to evolutionary
286 changes in a key reproductive trait that is regulated by variation in the same developmental
287 mechanisms operating in the model species *D. melanogaster*.

288

289 **Materials and Methods**

290 Hawai'ian *Drosophila* were collected (69) at the Koke'e State Park and Kui'a NAR on
291 Kauai, West Maui Watershed Reserve, Makawao Forest Reserve, and Waikamoi Nature Preserve
292 on Maui, and the Volcanoes National Park and Upper Waiakea Forest Reserve on Hawai'i
293 island. Field-caught flies were brought back to the lab for species identification and phenotyping
294 of adult and larval characters. Measurements of adult ovariole number, larval TF and TFC
295 number were performed as previously described (29). Mature egg size and adult body size were
296 quantified from white light micrographs of eggs and adult thoraces using ImageJ. See
297 Supplementary Information for detailed methods.

298 We combined sequence data for 18 genes reported in four previous studies (44, 46, 48,
299 53) from GenBank with additional newly identified mitochondrial sequences (Table S9), and
300 used the concatenated sequences to generate trees in RAxML v8.2.3 (70). Phylogenetic
301 relationships and divergence time estimates were inferred in a Bayesian framework in BEAST v.

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302 2.3.2 (71, 72). All phylogenetic comparative analyses and corresponding figures were computed
303 in R version 3.2.0 (73).

304 We used reported ecological information about Hawai'ian *Drosophila* to code oviposition
305 site (41), calculated ancestral states for each of these character codings with BEAST using the
306 *rayDISC* function in the R package **corHMM**, v.1.18 (74), mapped the most likely ecological
307 state at each node, and pruned the resulting tree to include only tips with ovariole number data.
308 The fit of different models of trait evolution was assessed on the pruned trees in **OUwie** v.1.48
309 (75). See Supplementary Information for detailed methods and custom scripts.

310

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- 487

488

489 **Figure Legends**

490

491 **Figure 1. Reproductive and ecological traits of Hawai'ian *Drosophila* in phylogenetic**

492 **context.** Compiled adult life history traits (greyscale gradients) collected herein and by

493 Kambysellis and Heed (42) are mapped on a phylogeny of Hawai'ian *Drosophila* constructed

494 from available mitochondrial and nuclear genes. Egg-laying substrate of each species is indicated

495 by colored boxes: bark (brown), generalist (black), sap flux (yellow), leaf (green), fungus

496 (purple), fruit (red), spider eggs (blue), flowers (pink), and unknown (gray). Boxes with solid

497 outlines denote data collected in the present study; boxes with four notches denote data

498 represented in our data and those of Kambysellis and Heed (42); boxes with dotted outline

499 denote data represented only in Kambysellis and Heed (42). Missing boxes indicate data points

500 that were either not previously reported (42) or that we were unable to obtain from field-caught

501 samples. Black lines at right delineate the five major groups of Hawai'ian *Drosophila* as follows:

502 SCAP = *Scaptomyza*; PW = picture wing; MM = modified mouthparts; H = Haleakala; AMC =

503 antopocerus-modified tarsus-ciliated tarsus.

504

505 **Figure 2. Different ecological states tested for OU analysis.** (A) A two-state model (OU2) of

506 bark-breeders (brown) and non-bark breeders (white). (B) Three-state model (OU3) that codes

507 bark-breeders (brown), spider egg and flower breeders (blue), and other oviposition substrates

508 (white). (C) Eight-state model (OU8) that codes each egg-laying substrate separately, color

509 coded as in Figure 1. Pie charts show the maximum likelihood ancestral state estimates at each

510 node, calculated with the rayDISC function in the R package **corHMM**, v.1.18 (74).

511

512 **Figure 3. Allometric relationship between life history traits in Hawai'ian *Drosophila*.**

513 Scatter plots of log transformed adult measurements with phylogenetically transformed trend
514 lines generated by averaging runs from PGLS analysis across 100 posterior distribution BEAST
515 trees, performed with the R package **nlme** v.3.1-121 (76). Trend line of the consensus tree is
516 denoted in red when there was a significant relationship between the two traits, and black when
517 PGLS analysis did not support a significant relationship (Table 2). (A, A1-A4) Ovariole number
518 plotted against thorax volume (mm³) in (A) all specimens, (A1) PW, (A2) MM, (A3) AMC, and
519 (A4) *Scaptomyza*. (B, B1-B4) Egg volume (μm³) plotted against thorax volume (mm³) in (B) all
520 specimens, (B1) PW, (B2) MM, (B3) AMC, and (B4) *Scaptomyza*.

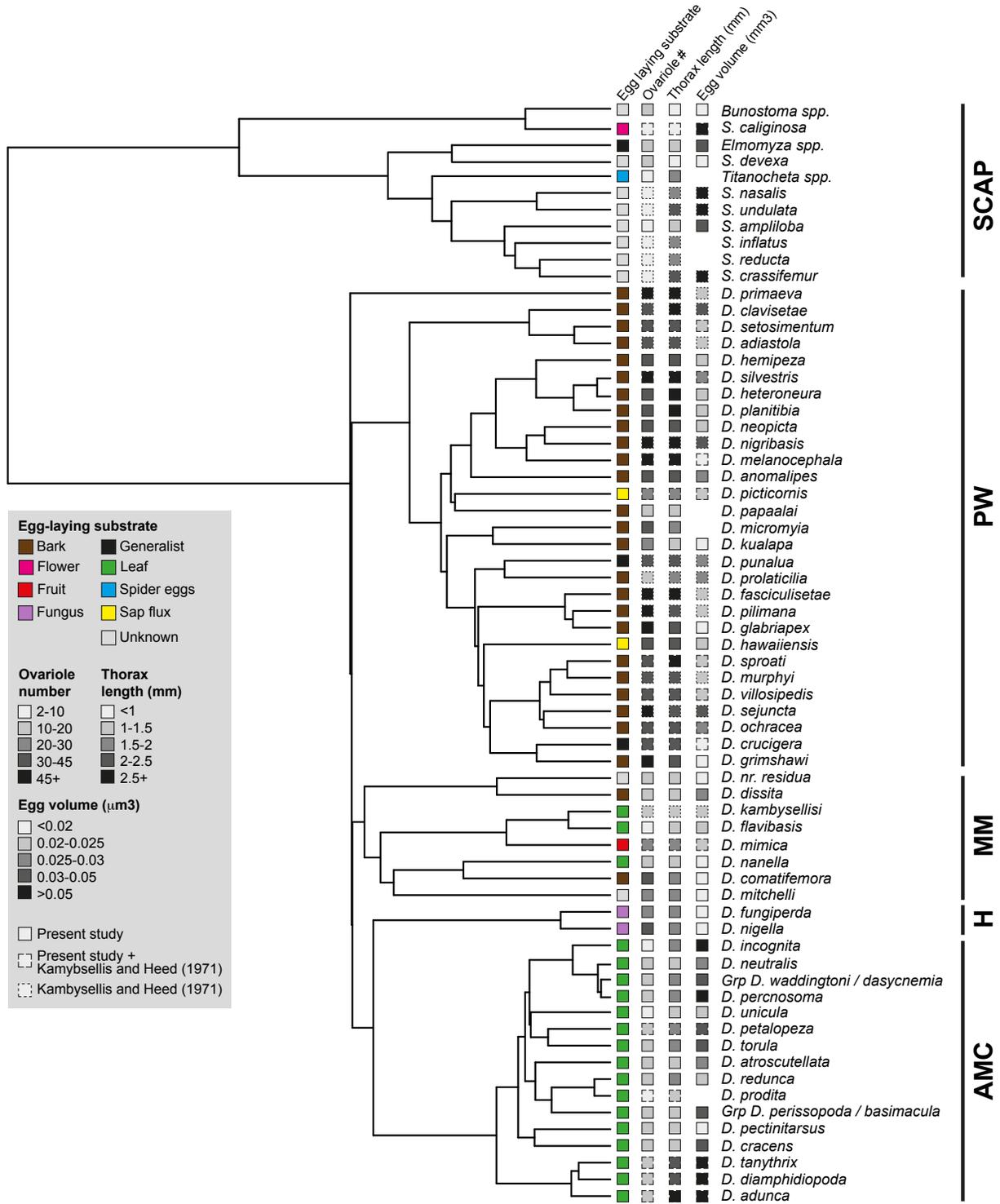
521

522 **Figure 4. Terminal filament cell (TFC) number predicts terminal filament (TF) number in**

523 **Hawai'ian Drosophilids.** (A-C) Bar graphs for (A) TFC number per TF, (B) total TFC number,
524 and (C) TF number per larval ovary representing the mean and standard deviation, as well as the
525 phylogenetic relationship between the species shown (bottom). (D-F) Late third instar larval
526 ovaries stained for nuclei (purple) and F-actin (green) for (D) *S. caliginosa* (flower breeder), (E)
527 *D. silvestris* (bark breeder), (G) *D. mitchelli* (egg-laying substrate unknown), and (F) *D.*
528 *tanythrix* (leaf breeder). Numbers in parentheses beside species names indicate mean ovariole
529 number per ovary (Tables S7, S8). White arrowheads indicate TF structures in the ovary.

530

531 **Figure 1**

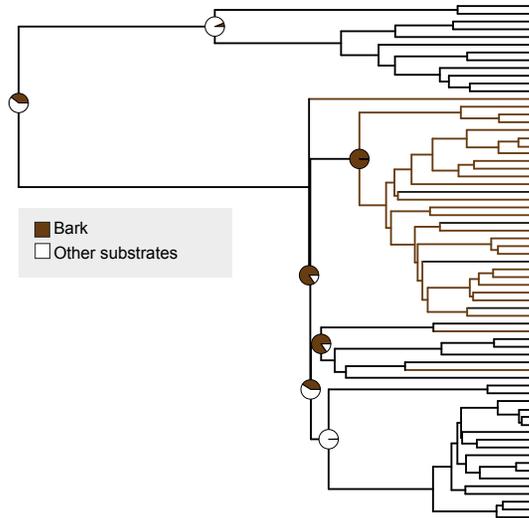


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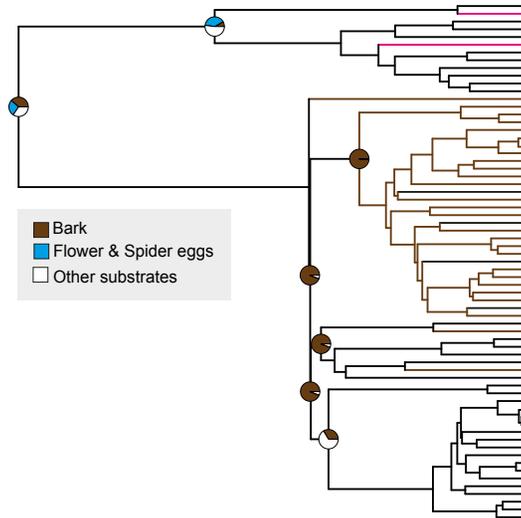
533 **Figure 2**

534

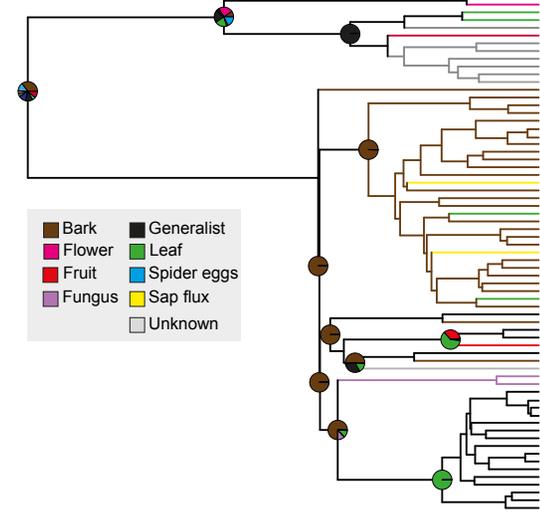
(A) Two state (OU2)



(B) Three state (OU3)

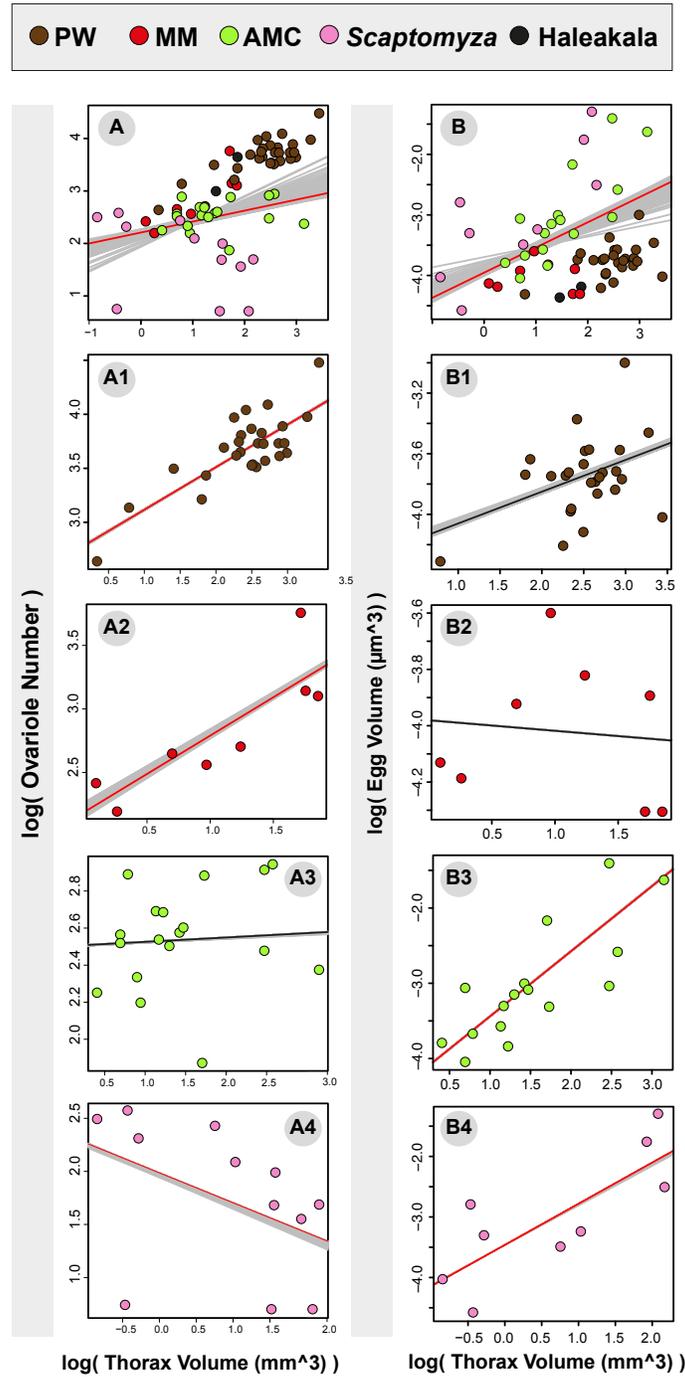


(C) Eight state (OU8)



535

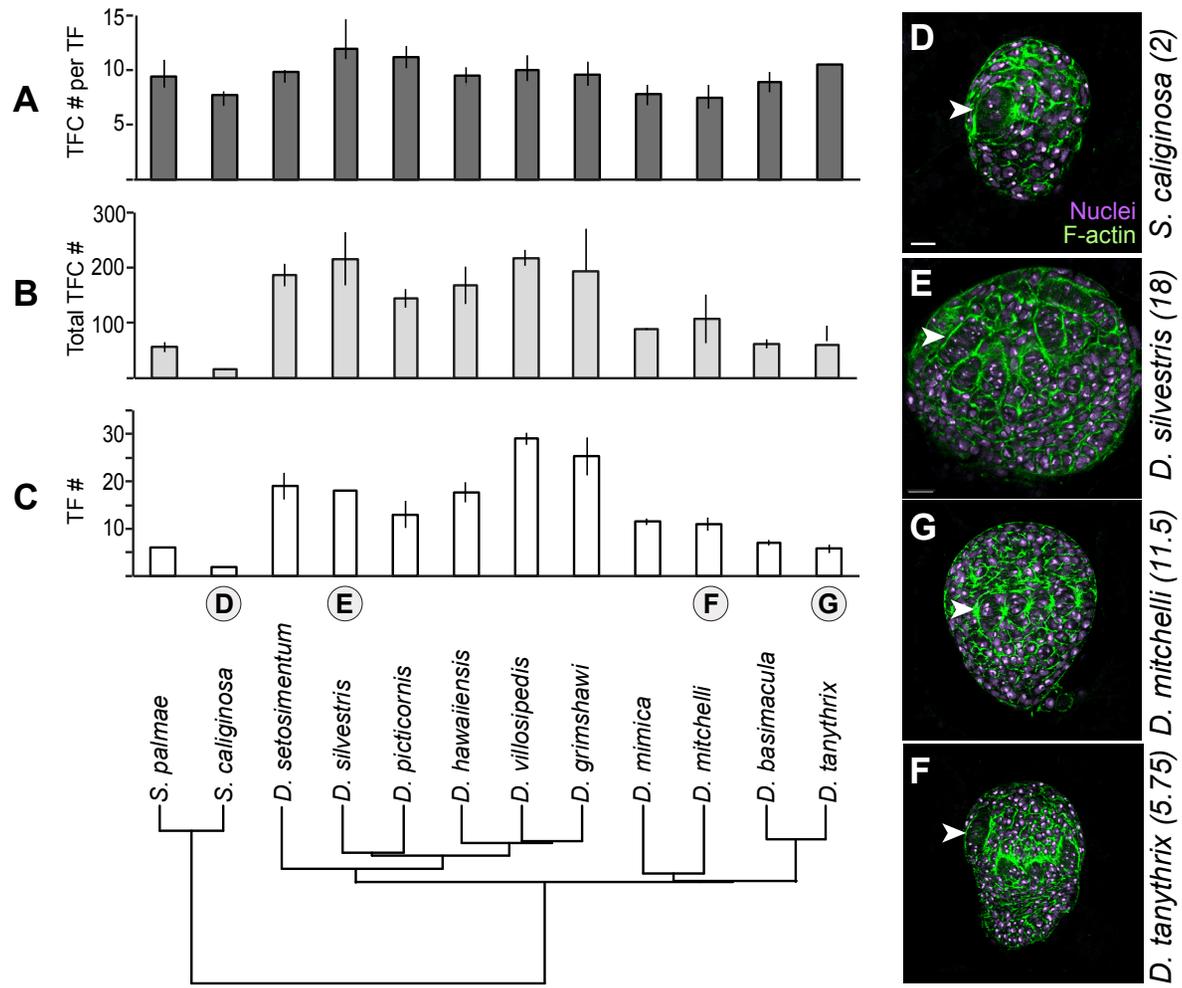
536 Figure 3



537

538 **Figure 4**

539



540

541 **Table 1. Comparison of AICc and weighted AICc values for models testing the relationship**
542 **between oviposition substrate and ovariole number.** Values are for model fit of Brownian
543 motion (BM) and Ornstein-Uhlenbeck with one optimum (OU1) or with multiple optima (OUM)
544 with different combinations of oviposition substrate categories, calculated with the R package
545 **OUwie** v.1.48 (75). Oviposition substrates were categorized as follows: OU2 categorizes species
546 that lay eggs on bark and non-bark; OU3 categorizes species into bark-breeder, spider egg/flower
547 breeder, and other; and OU8 categorizes each species according to the eight oviposition
548 substrates represented (bark, flower, spider egg, fruit, leaf, generalist, fungus, sap flux). Models
549 were tested over 1000 posterior distribution BEAST trees using nuclear and mitochondrial gene
550 sequences. Bold indicates the best supported model.
551

	AICc	Δ AICc	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

552

553

554 **Table 2. Phylogenetic Generalized Least Squares (PGLS) analysis of adult reproductive traits in Hawai'ian *Drosophila*.** PGLS
 555 analysis of relationships between ovariole number and thorax volume (mm³), egg volume (μm³) and thorax volume, and ovariole
 556 number and proportional egg volume (μm³/mm³) are listed. Regression analyses were performed with the R package **nlme** v.3.1-121
 557 (76) on 100 trees from a BEAST posterior distribution using nuclear and mitochondrial genes, and the minimum, average, and
 558 maximum slope and p-value for the analysis is included in the table. P-values below 0.01 are indicated in bold.
 559

		All species groups			PW spp.			AMC spp.			MM spp.			Scaptomyza spp.		
		min	avg	max	min	avg	max	min	avg	max	min	avg	max	min	avg	max
ON - Thorax volume (mm ³)	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	-	-	-
	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 volume (μm ³) - Thorax volume (mm ³)	Slope	0.156	0.353	0.407	0.164	0.185	0.164	0.745	0.748	0.760	-	-	-	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 (μm ³ /mm ³)	Slope	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	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 (μm ³)	Slope	0.703	-0.42	0.376	0.088	0.081	-0.07	0.308	0.2224	0.161	0.689	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

560
561

562 **Table 3. Phylogenetic Generalized Least Squares (PGLS) analysis of larval ovarian**
563 **measurements in Hawai'ian *Drosophila*.** Relationships between TF number and TFC number
564 per TF, TF number and total TFC number, and total TFC number and TFC number per TF are
565 listed. Regression analyses were performed with the R package **nlme** v.3.1-121 (76) on 100 trees
566 from a BEAST posterior distribution using nuclear and mitochondrial genes, and the minimum,
567 average, and maximum slope and p-value for the analysis is included in the table. P-values below
568 0.01 are indicated in bold.
569

		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

570