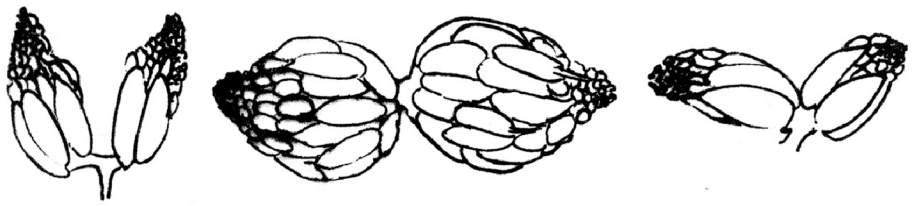
ISSN 0014-3820

EVOLUTION

INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

VOL. 54 OCTOBER 2000 NO. 5



Drosophila melanogaster *Drosophila sechellia*

low food high food high food

Intraspecific plasticity and interspecific variation in ovary size in Drosophilids • Jason Hodin and Lynn Riddiford, p. 1638.

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PUBLISHED BY THE SOCIETY FOR THE STUDY OF EVOLUTION

Evolution, 54(5), 2000, pp. 1638–1653

DIFFERENT MECHANISMS UNDERLIE PHENOTYPIC PLASTICITY AND INTERSPECIFIC VARIATION FOR A REPRODUCTIVE CHARACTER IN DROSOPHILIDS (INSECTA: DIPTERA)

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Abstract.—The insect ovary is a modular structure, the functional unit of which is the ovariole. Ovariole number is positively correlated with potential reproductive output. Among drosophilids (Insecta: Diptera), ovariole number shows both phenotypic plasticity and substantial interspecific and interpopulational variation. Here we examine the mechanistic connection between phenotypic plasticity and genetically fixed variation in ovariole number within the *melanogaster* species group. When a laboratory population of *Drosophila melanogaster* was reared under reduced food conditions, differences in ovariole number were entirely due to alterations in cell differentiation during the wandering stage at the very end of larval development. Cell growth and cell death were not affected. When these same flies were reared under a variety of temperatures, ovariole number differences arose during the latter half of the third (final) larval instar. Cell differentiation was affected, although cell number was not, and ovariole number differences were established before metamorphosis. In contrast, genetically fixed, interspecific and interpopulational variability in ovariole number was caused by alterations in the dynamics of cell differentiation and by cell number differences. Furthermore, the stages affected were different in different species and populations in the *melanogaster* species group, ranging from the first (*D. sechellia*) through the middle of the third (*D. simulans* and *D. mauritiana*) larval stage. Therefore, the mechanistic bases for plasticity-based variability are largely distinct from the mechanistic bases for interspecific and interpopulational variability. Our results suggest that phenotypic plasticity indicates evolutionary flexibility in underlying ontogenetic processes.

Key words.—Development and evolution, *Drosophila mauritiana*, *Drosophila melanogaster*, *Drosophila sechellia*, *Drosophila simulans*, *Drosophila yakuba*, ovariole number, phenotypic plasticity.

Received August 2, 1999. Accepted March 9, 2000.

What is the relationship between phenotypic plasticity and interspecific or interpopulational variation? Speculations on this subject range from the hypothesis that plasticity imposes limits on the process of natural selection (thus reducing variation) to the theory that plasticity can help maintain genetic variation in the absence of selection (reviewed in Schlichting and Pigliucci 1998). Alternatively, although not mutually exclusively, plasticity and interspecific variation can be viewed as inherently developmental phenomena: plasticity as ontogenetic variability within a genotype due to environmental factors and interspecific variation as genetically fixed differences in ontogenetic trajectories between genotypes. Under this conception, a determination of the relationship between plasticity and interspecific variation involves a comparison of the ontogenetic trajectories leading to the production of a morphological character via plastic and genetically fixed routes. Are the developmental mechanisms underlying a plastic response in a trait the same as the developmental mechanisms underlying interspecific variation for that same trait? If they are the same, a direct link between plasticity and genetically fixed variation is likely; if they are different, either a more complex relationship or no relationship exists.

Few studies have compared the developmental mechanisms underlying plasticity to the developmental mechanisms underlying genetically fixed variability. Partridge et al. (1994) demonstrated that environmentally induced increases in wing size in *Drosophila melanogaster* had the same mechanistic basis (increased cell size) as the increase in wing size in artificially selected lines. Yet, de Moed et al. (1997) found that interpopulational variation in wing size in *D. melanogaster* depended largely on differences in cell number rather than cell size. Brakefield et al. (1996, 1998) showed that the mechanistic bases for seasonally variable eyespot size on the ventral forewings of satyrine butterflies were mirrored in lines selected for constitutive production of either large or small eyespots. In contrast, Rountree and Nijhout (1995a,b) found that distinct ontogenetic mechanisms led to the production of the dark-wing phenotype in plastic versus constitutively dark-winged populations of the buckeye butterfly. Yet, no large-scale study involving several closely related species expressing substantial variation for a given trait has been done.

Ovarioles are the functional units of the insect ovary. They are essentially assembly lines for the production of eggs, allowing a female with multiple ovarioles to mature multiple eggs simultaneously. In this way, ovariole number correlates with potential reproductive output and, thus, fitness (David 1970; Cohet and David 1978; Boulétreau-Merle et al. 1982). Trade-offs may exist for insects with the large ovaries characterized by high ovariole numbers, including possible costs to flight maneuverability (Berrigan 1991) or developmental production costs. Ovariole number is relatively variable in drosophilids (Mahowald and Kambysellis 1980), and in Hawaiian drosophilids is associated with oviposition substrate (Kambysellis and Heed 1971; Kambysellis et al. 1995). Even within the *melanogaster* species group (Fig. 1), substantial variability exists: whereas most *D. melanogaster* populations are characterized by ovariole numbers on the order of 18–23 per ovary, the island species *D. sechellia* has only eight or

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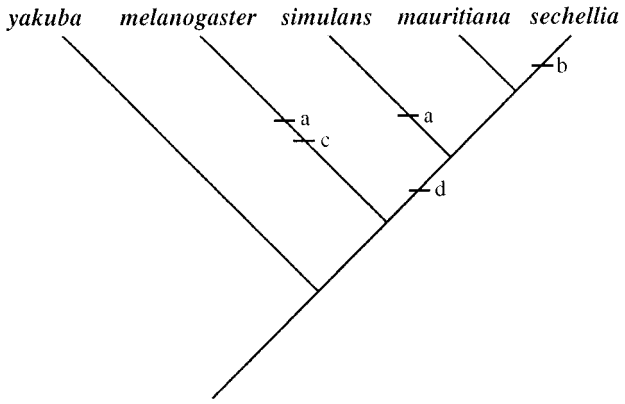

FIG. 1. Phylogeny of the *melanogaster* species group (after Caccone et al. 1996). Mapped onto this phylogeny are possible reconstructions of evolutionary increases (a) and decreases (b) in ovariole number. Under this hypothesis, the ancestral condition was a mean ovariole number of approximately 13 ovarioles per ovary (but see the Discussion). The letter c represents the apparent evolutionary increase in primordium size in *melanogaster*; d represents an apparent evolutionary decrease in cell size in early third larval instar ovarian primordia in the *simulans-mauritiana-sechellia* clade (see the Discussion). Character state reconstructions were done by hand.

nine ovarioles per ovary. This unusual species has evolved the ability to feed on the toxic fruit *Morinda citrifolia* (Louis and David 1986) and therefore avoids interspecific competition. The other species in the *melanogaster* group have mean ovariole numbers between *D. melanogaster* and *D. sechellia*. Substantial variation among populations also exists for this character in *D. melanogaster* (Pappas and Engstrom 1974). In addition, there is well-documented phenotypic plasticity for ovariole number within different populations of *D. melanogaster* when the larvae are raised over a broad temperature range (Delpuech et al. 1995; Morin et al. 1997; Moreteau et al. 1997), in crowded rearing conditions (Robertson 1957), or with restricted larval nutrition (Savilev 1928).

The ontogenetic mechanism of determination of ovariole number has only been studied in honeybees. At the end of the penultimate larval instar, the ovaries of queens and workers appear identical, both beginning to form hundreds of ovarioles (Zander et al. 1916). In the final instar, the ovaries of workers undergo massive cell death, resulting in adult ovaries with generally fewer than 10 ovarioles per ovary (Zander et al. 1916; reviewed in Ruttner 1988), whereas queens' ovaries continue to mature (Hartfelder and Steinbrück 1997). Whether cell death is a general mechanism underlying interspecific and interpopulational differences in ovariole number in insects is not known.

Here we take a developmental approach to compare plasticity-based and genetically fixed variability in ovariole number in the *melanogaster* species group. Because ovariole number is determined during the final larval stage (King et al. 1968; Godt and Laski 1995; Hodin and Riddiford 1998), we compare the trajectories of ovarian growth and differentiation in the larval ovarian primordia of these species. Ovarian differentiation begins with the formation of the terminal filaments (TFs), pancake-like stacks of somatic cells that will ultimately cap the apical end of each ovariole (King et al. 1968; Godt and Laski 1995). The process of TF formation is complete at pupariation (King et al. 1968; Godt and Laski 1995; Hodin and Riddiford 1998), the onset of metamorphosis. Thus, differences in ovariole numbers in newly eclosed adult flies (either genotypic or phenotypic) may be either due to differences in the numbers of TF stacks at pupariation or due to removal, by cell death, of TF stacks or precursors during larval and/or pupal development.

For the four different species in the *melanogaster* species group, as well as for *D. yakuba*, a closely related species (Lachaise et al. 1988) that we examined as an outgroup (Fig. 1), we compare the ontogenetic bases of interspecific and interpopulational (genetically fixed) variation to plasticity in *D. melanogaster* larvae reared under a variety of food and temperature conditions. The finding that ontogenetic processes that vary among species and populations are identical to the ontogenetic processes that are altered when flies of a single population are reared under divergent environmental conditions would support the theory that plasticity and genetically fixed variation are intimately connected. However, if the ontogenetic basis for genetically fixed variation were distinct from the ontogenetic basis for plasticity-based variability, then there may be either no connection or a more complex interaction between plasticity and genetically fixed variation. Our results indicate that phenotypic plasticity implies evolutionary flexibility in underlying ontogenetic mechanisms.

MATERIALS AND METHODS

Fly Stocks

Three strains of *D. melanogaster* were used: the Sevelen strain, collected in Sevelen, Switzerland, and maintained in the laboratory for more than 40 years (a gift of G. Schubiger); the Capitol Hill strain, collected in Seattle, Washington, in 1996 by J. Hodin; and the Nahal Canyon 120m strain, collected at that elevation on the north slope of Nahal Canyon, Israel, by T. Pavlicek in 1994. *Drosophila mauritiana* was collected in Riviere Noire, Mauritius, in 1973 by J. David (Bowling Green Species Center stock number 14021–0241.0). *Drosophila sechellia* was collected on Cousin Island in the Seychelles Islands in 1981 by F. Lemeunier (Bowling Green Species Center number 14021-0258.1). *Drosophila yakuba* was collected in the Ivory Coast, Africa, by H. Burla (Bowling Green Species Center stock number 14021-0261.0). Most *D. simulans* experiments (unless noted) used a *D. simulans* strain carrying an st^1 eye mutation, which arose in an isofemale line collected in 1985 in Florida City, Florida, by J. Coyne (Bowling Green Species Center stock number 14021-0251.32). Two other *D. simulans* strains were used: one collected in Lima, Peru, in 1953 by W. Heed and Cowan (Bowling Green Species Center stock number 14021-0251.5), the other collected in Zamorano, Honduras, in 1954 by W. Heed (Bowling Green Species Center stock number 14021-0251.3). Fly stocks were maintained at 18°C in vials on standard *Drosophila* medium: 9% light, unsulfured molasses (Sam Wylde Flour Co., Seattle, WA); 9% w/v yellow, degerminated cornmeal (Sam Wylde); 3.5% w/v brewers yeast (ICN Biochemical, Costa Mesa, CA); 0.8% w/v fine ground agar (Morehead and Co., Van Nuys, CA); and 1.2% Tegosept solution (66 mM methyl 4-hydroxybenzoate [Aldrich Chem-

ical Co.] in 95% ethanol, a mold inhibitor) in tap water. Unless noted, all experiments were performed at 25°C, and the flies were kept in these conditions for at least two generations before any experiments were done. ''Half food'' is one part standard *Drosophila* medium to one part 3% w/v agar (Difco Laboratories, Detroit, MI). The total volume of food was the same as in full food bottles or vials.

Staging

Flies were allowed to lay eggs on fresh food for at least 1 h to limit overaged embryos before collecting. To obtain ovaries at 24 h after hatching, embryos were collected on grape juice agar plates, seeded with a few drops of acetic acid (or 1% n-caproic acid for *D. sechellia*) and yeast, selected at 0–2 h after hatching, transferred to microwaved *Drosophila* medium in vials (~50 larvae/vial), aged for 24 h, and dissected.

To obtain third larval instar ovaries (before wandering stages), approximately 200 eggs were collected on microwaved (softened) food in standard *Drosophila* pint bottles that had been split horizontally about 4 cm from the bottom (this allows for easy egg counts) and aged for about 64 h. Late second instar larvae were then transferred to yeast paste (yeast + distilled water) and selected 0–2 h after ecdysis to the third instar. Larvae were then either dissected or transferred to microwaved *Drosophila* medium in vials (~50 larvae/vial), and aged until the desired stage had been reached.

To obtain ovaries at wandering stages, at pupariation and in adults, approximately 200 eggs were collected in split, standard *Drosophila* pint bottles. Early-wandering larvae were aged for approximately 100 h until the larvae first start emerging from the food and initiated wandering behavior. Animals were selected 0–3 h after the initiation of wandering, and only animals with full guts were dissected (larvae purge their gut contents in the course of the approximately 10 h of wandering). Late-wandering larvae had empty guts, and many of their cohorts had already pupariated. Pupariation-staged animals were selected 0–2 h after pupariation (the onset of metamorphosis), when the animals ceased wandering to form puparia. To eliminate the possible effects of adult nutrition on ovariole number (Chippindale et al. 1993), adults were well fed after eclosion until the time of dissection (3–5 days after eclosion).

The 15°C, 18°C, and 30°C experiments were performed as follows: Eggs were collected as described above, and the bottles were transferred to and maintained in incubators at the appropriate temperature until dissection. Adult dissections were performed on flies that had been transferred to 25°C at eclosion and treated as described above.

Dissections and Immunocytochemistry

Larvae and pupae were dissected and fixed as previously described (Hodin and Riddiford 1998), except that the fixative included 1% Triton X-100. Brains from wandering larvae were used as controls and were processed together with the ovaries.

Ovaries to be treated with antibodies were first blocked for at least 30 min at room temperature in 5% normal goat serum in PBT (phosphate buffered saline PBS: 130 mM NaCl, 10 mM $NaPO_4$, pH 7.0] with 1% Triton X-100) with 0.01% sodium azide, and then incubated in *Drosophila* USP monoclonal antibody (1:300 AB11; Khoury-Christianson et al. 1992) diluted in PBT with 0.01% sodium azide for either 3 h at room temperature or overnight at 4°C. Next, ovaries were washed three times for 20 min in PBT, and incubated in 1:1000 donkey anti-mouse FITC, Texas red, or CY5 (Jackson ImmunoResearch, West Grove, PA) secondary antibody in PBT with 0.01% sodium azide in the dark for either 3 h at room temperature or overnight at 4°C: Ovaries were then washed three times for 20 min in PBT and, if required, double-labeled with Bodipy-phalloidin 558/568 (1 unit/200 μl PBT; Molecular Probes, Eugene, OR) for 45 min at RT to visualize cortical actin (cell borders), and then washed three times for 20 min in PBT. Ovaries were mounted in 90% glycerol/1× PBS/0.01% sodium azide for confocal microscopy.

Primordium Size

Ovaries dissected 0–2 h after ecdysis to the third instar were fixed and stained with phalloidin as described above. We measured length and width of the ovary in one central confocal section using the scale bar function in the COMOS confocal software package (BioRad, Hercules, CA). For calculations of cross-sectional area, the ovary at this stage was assumed to be an oblate spheroid (yielding an elipse in cross section).

Total Cell Number Counts

Ovaries of the appropriate stage were dissected, washed in PBS, then transferred to dissociation medium (30 mM EGTA, 1.5 mg/ml collagenase I [Worthington Biochemicals, Lakewood, NJ, #4174, 276 U/mg], 10 μg/ml Hoechst 33258 [Sigma, St. Louis, MO], in PBS) in a 96-well plate and gently shaken for 2–4 h at room temperature. Ovaries were then carefully transferred to a slide and flattened under a cover slip. This creates a monolayer of cells that is visible under UV light (as the nuclei are labeled by the Hoechst stain). The ovaries were examined using a Nikon HFX-IIA fluorescent microscope, and the images captured using a Sony 3CCD color video camera and the video capture features in NIH Image Software (National Institutes of Health, Bethesda, MD) or Video Player (Apple, Cupertino, CA). Images were then processed in Adobe Photoshop, printed on a LaserWriter 12/640 PS (Apple), and the numbers of cells counted by hand.

Cell Death

Ovaries of the appropriate stage were dissected, fixed, and blocked as described above, and then transferred to 96-well plates with 4 μl of 5× terminal deoxynucleotide transferase (TdT) buffer (250 mM potassium cacodylate [pH 7.2], 5 mM $CoCl_2$, 0.5 mM DTT; Gibco-BRL, Gaithersburg, MD) diluted in 16 μl PBT for at least 10 min. Ovaries were then incubated in 20 μl of TdT reaction mixture (1× TdT buffer, 1% Triton X-100, 0.067 mM dUTP, 0.033 mM biotin-dUTP, 0.33 U/μl TdT [Gibco-BRL] in dH_2O) for 3 h at 37°C, then transferred back to staining chambers, washed 6 times for 10 min in PBT, and incubated in 1:500 FITC-conjugated streptavidin

(Jackson) secondary antibody in PBT with 0.01% sodium azide for 3 h at room temperature or overnight at 4°C. Samples were washed and mounted as described above.

Cell death indices were calculated for these TdT-labeled ovaries by averaging the total numbers of labeled cells per ovary (counted on a Bio-Rad 600 confocal microscope) for each stage and dividing by the average total numbers of cells in ovaries of that stage (as described above). Note that the cell death and cell number counts were, of necessity, performed on different ovaries, and that our analysis assumes equal weighting of the two samples across the size range.

Terminal Filament and Ovariole Number

Ovaries of the appropriate stage were dissected, fixed, and immunostained with the USP antibody, which strongly labels forming terminal filament (TF) cells in *D. melanogaster* (Hodin and Riddiford 1998) and further processed as described above. Ovaries at pupariation were stained with phalloidin to count the fully formed TFs. Samples were then analyzed by confocal microscopy, and the total numbers of incipient (or fully formed) TF stacks were counted. To count adult ovariole numbers, the ovaries were removed from etherized flies, and the ovarioles teased apart with tungsten needles and counted under a dissecting microscope.

Pupal Dry Weights

Five pupae for each condition were dried individually at 50°C for two to three days in preweighed, aluminum-foil weigh boats placed in petri plates containing a few desiccant stones. Pupae were weighed using a Mettler M5 analytical balance (Mettler Instruments, Toledo, OH).

Cell Size

For the calculations of mean ovarian volumes at the onset of the third larval stage, we used the data from primordium size (see above) and assumed that the ovary is an oblate spheroid. Thus, volumes were calculated using the formula: $3/4\Pi(r^1)(r^1)(r^2)$, where r^1 was the smaller radius in cross section for these ovaries. When plotted against mean total cell number (see above), the resulting graph yields information about cell size (see legend to Fig. 7E).

Confocal Microscopy and Image Processing

Confocal images were obtained using a BioRad MRC-600 Confocal laser scanner, an Optiphot Nikon microscope, and COMOS software (BioRad). Images were processed using NIH Image (National Institutes of Health) and Adobe Photoshop (Adobe Systems).

Statistics

ANOVA, *t*-tests, and standard error calculations were performed using the Systat 5.2.1 software package (Systat, Chicago, IL). Where appropriate, sequential Bonferroni adjustments for multiple comparisons were performed, as described by Rice (1989). In most cases, the *n*-values reported here reflect numbers of animals dissected, one ovary per animal. However, several of the terminal filament (TF) counts included data in which both ovaries had been dissected from each animal. In these cases (Sevelen full food AP and 24 h after ecdysis to the third instar; Sevelen half food AP and 24 h and 30 h after ecdysis to the third instar; *D. mauritiana* 24 h after ecdysis to the third instar; *D. simulans-st* AP and 30 h after ecdysis to the third instar; *D. sechellia* AP; and Capitol Hill full and half food AP), at least 20 ovaries from at least 10 animals were analyzed. We chose 10 ovaries at random in 10 separate replicates from this pool, and calculated means, standard errors, and performed *t*-tests on all 10 replicates. The values reported are means of the 10 replicate analyses.

Regression analyses and curve fitting were performed using the Cricket Graph 1.0 software package (Computer Associates International, Islandia, NY). *P*-values for the regression analyses are from the critical values tables in Zar (1984).

The wandering-stage TF formation rate comparisons were calculated as described in Sokal and Rohlf (1981) using their simplified equations for calculating the standard errors of the differences between two means. It should be noted that the significance estimates calculated this way were extremely conservative. Because we could not compare TF numbers at wandering and pupariation in the same animals, our variance estimates for TF formation rates were undoubtedly amplified.

Ideally, to compensate for possible confounding effects of body size on the ovarian ontogenetic variables tested (e.g., cell number, ovariole number), one should perform an analysis of covariance (Cochran 1957; Finney 1957; Cox 1958). However, because the animals used for our body size (pupal weight) measurements were, of necessity, different than the animals used to measure the other ontogenetic variables, a proper covariance analysis could not be done. Therefore, we have used the admittedly inferior method of dividing test variables by mean pupal weight, realizing that this assumes an isometric scaling of these variables with body size. We have no data to support this assumption.

RESULTS

In drosophilids, the ovarian primordium is established during embryogenesis (reviewed in Williamson and Lehmann 1996), then grows throughout larval life (Kerkis 1931, 1933). In the third larval instar (final larval stage), the TF stacks are formed progressively (King et al. 1968; Godt and Laski 1995; Hodin and Riddiford 1998), so that at the time of pupariation the maximal ovariole number is fixed by the number of TF stacks present. Ovariole number might then be reduced either by cell death during subsequent metamorphic development or after eclosion, depending on age, temperature, or nutrient availablity (Cohet and David 1978; Chippindale et al. 1993; Huey et al. 1995; Carlson et al. 1998).

Interspecific and Interpopulational Variability in Ovariole Number and the Effects of Different Environmental Conditions

Figure 2A shows the variability in adult ovariole numbers in the different members of the *melanogaster* species group and the *D. yakuba* outgroup, reared in uncrowded conditions at 25°C on an excess of standard *Drosophila* laboratory food.

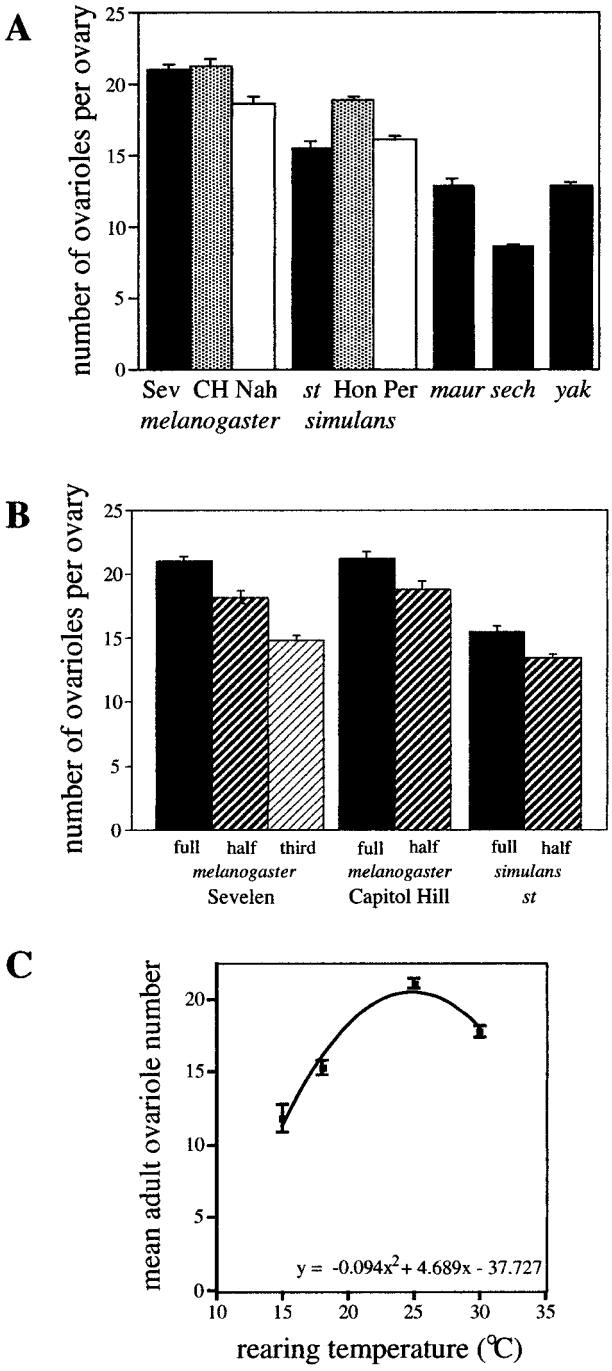

FIG. 2. Adult ovariole numbers. Values are means of 20 ovaries dissected from 10 adults, 3–5 days after eclosion. (A) Interspecific and interpopulational variation in ovariole number, in three populations of *Drosophila melanogaster* (Sev, Sevelen; CH, Capitol Hill; Nah, Nahal), three populations of *D. simulans* (Hon, Honduras; Per, Peru), and one each of *D. mauritiana* (*maur*), *D. sechellia* (*sech*), and *D. yakuba* (*yak*, the outgroup). (B) Effects of reduced larval nutrition on ovariole number. One-third-food treatments result in developmental delays, whereas half-food treatments do not. Sevelen and Capitol Hill are *D. melanogaster* populations. (C) Ovariole number as a function of rearing temperature (norm of reaction) in *D. melanogaster*-Sevelen ($y = -0.094x^2 + 4.689x - 37.727$). All error bars are standard errors.

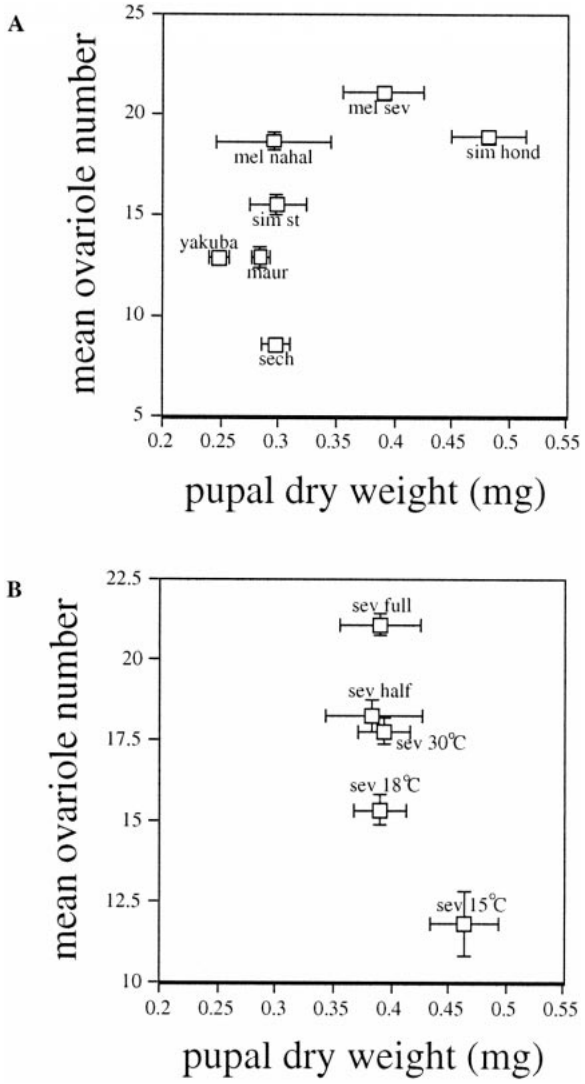

FIG. 3. Mean adult ovariole number ($n = 20$ ovaries, 10 animals) as a function of pupal weight ($n = 5$ pupae each). (A) There is substantial interspecific and interpopulational variation in both body size and ovariole number, but the correlation is not significant ($r = 0.583$, $P > 0.1$). (B) There is no correlation between body size and ovariole number in *Drosophila melanogaster* larvae reared under various temperature and nutrient conditions ($P = 0.1$). Abbreviations as in Figure 2A. All error bars are standard errors.

Mean ovariole numbers per ovary ranged from 8.5 in *D. sechellia* to 21 in the *D. melanogaster*-Sevelen (Switzerland) and Capitol Hill (Seattle) strains. One wild-caught strain of *D. melanogaster* from Nahal Canyon in Israel had a lower mean ovariole number (18.5). Two geographically distinct populations of *D. simulans*, the *st* strain (Florida City) and a Peruvian population, had about 16 ovarioles, whereas a Honduran population had a mean of about 19 ovarioles. The island species *D. mauritiana* and our outgroup, *D. yakuba*, had 13 ovarioles per ovary.

To determine if these differences in ovariole number were simply due to differences in body size, we measured pupal dry weights for the various species. Figure 3A shows that there are significant differences across species in ovariole

number among pupae of the same size. Within species (between populations), however, there was substantial variability (ANOVA) in both mean ovariole number (*D. melanogaster*: $P = 0.004$; *D. simulans*: $P < 0.001$) and pupal dry weight (*D. melanogaster*: $P = 0.007$; *D. simulans*: $P < 0.001$). Furthermore, the two strains with the significantly higher pupal weights (*D. melanogaster*-Sevelen and *D. simulans*-Honduras) also had higher ovariole numbers. Thus, body size differences could explain much of the interpopulational variability that we might detect in parameters of ovarian growth and differentiation.

Developmental rates were fairly consistent among species. *Drosophila simulans, D. yakuba,* and *D. melanogaster* populations developed at essentially the same rate, whereas *D. sechellia* and *D. mauritiana* were much less synchronous, and larvae resynchronized at ecdysis to the third instar had a slightly longer third instar period than the other species (approximately 6–8 h longer; data not shown).

Drosophila melanogaster-Sevelen and *D. simulans-st* flies raised on a diet with half the nutrient level (henceforth, ''half food'') had significant reductions in adult mean ovariole number ($P < 0.001$ and $P = 0.015$, respectively; Fig. 2B). In contrast, the Capitol Hill strain of *D. melanogaster* on half food showed a reduction in ovariole number ($P < 0.04$) that was not significant after we performed Bonferroni adjustments for multiple comparisons (Rice 1989). Sevelen larvae raised under half-food conditions completed the second larval instar approximately 6 h before fully fed larvae, but the lengths of the third larval instars were the same (not shown). In contrast, Sevelen flies raised on one-third-nutrient conditions had a greatly reduced development rate (approximately 20% slower than either full- or half-food-reared larvae; data not shown) and had even fewer ovarioles than did half-food flies ($P = 0.001$; Fig. 2B). However, for ease of staging comparisons, we restricted further analysis to the half-food flies.

Rearing temperature also significantly affected mean ovariole numbers (Fig. 2C). *D. melanogaster*-Sevelen larvae raised at 15°C, 18°C, and 30°C had significantly fewer ovarioles than those raised at 25°C ($P < 0.001$). Because temperature greatly affects development rate, analyses of temperature effects were restricted to identifiable developmental stages, namely ecdysis to the third instar, early wandering, and pupariation. The 15°C flies developed 3.67 times slower, 18°C flies developed about twice as slow, and 30°C flies developed about 1.25 times faster than flies at 25°C (data not shown). Figure 3B shows that neither nutrient level nor rearing temperature significantly affected body size in pupae.

In summary, we found that there is substantial interspecific and interpopulational variation for ovariole number within the *melanogaster* species group, confirming previous reports (Papas and Engstrom 1974; R'Kha et al. 1997). We have also confirmed (see Savilev 1928; Delpuech et al. 1995; Morin et al. 1997; Moreteau et al. 1997) that larvae reared under various nutrient and temperature conditions exhibit substantial phenotypic plasticity for ovariole number and that the range of the plastic response approaches that of the naturally occurring variation within the species group.

Primordium Size

Is the size of the undifferentiated ovarian primordium in the final larval stage related to adult ovariole number? At the onset of the third larval instar, the cross-sectional areas of the ovaries of the *D. melanogaster*-Sevelen and Capitol Hill strains were significantly greater than any of the other four species (Fig. 4A; $P < 0.015$ in every case). Figure 4B shows that ovariole number is positively correlated with the size of the ovarian primordium at the onset of the third instar ($r = 0.873$, $P = 0.005$). *Drosophila simulans-st* deviated from this relationship by forming more ovarioles than expected from its size at this stage, whereas *D. sechellia* formed fewer than expected from its size (although, in both cases, the correlation was still significant). When the effects of body size (pupal weight) were removed, the positive relationship between ovariole number and primordium size was still significant ($r = 0.748$, $P < 0.025$; data not shown), for all species other than *D. yakuba.* Thus, there may be a substantial effect of body size on ovariole number in *D. yakuba.*

We also detected significant, interpopulational variation in primordium size (Fig. 4A; *D. melanogaster*-Sevelen vs. Nahal, $P = 0.004$; *D. simulans-st* vs. Honduras, $P = 0.0001$), which correlated with differences in adult ovariole number (Fig. 4B). However, when the substantial interpopulational effects of body size (pupal weight; see above) were removed, these differences in primordium size were no longer significant ($P = 0.294$ for *D. melanogaster*; $P = 0.054$ for *D. simulans*).

The Capitol Hill and Sevelen strains of *D. melanogaster* under half-food conditions and the Sevelen strain raised at 15°C, 18°C, and 30°C showed no differences in primordium size when compared to Sevelen raised on full food at 25°C (Fig. 4C; $P > 0.2$ in every case). Furthermore, there was no correlation between primordium size at the onset of the third instar and mean adult ovariole number under these conditions (Fig. 4D; $P = 0.5$). For example, Sevelen raised at 15°C have substantially fewer ovarioles than Sevelen raised at 25°C (12 vs. 21; $P < 0.001$; Fig. 2C), but their primordium sizes (2529 μm^2 versus 2510 μm^2; $P = 0.872$; Fig. 4C) did not differ at the onset of the third larval instar. Removing the effect of body size (pupal weight) had no qualitative effect on any of these results (not shown).

In summary, interspecific differences in ovariole number correlate with ovarian primordium size at the onset of the third larval instar. In contrast, plasticity-based ovariole number differences show no such correlation with ovarian primordium size at this stage.

Growth in Second Instar Larval Ovaries

To determine if the smaller ovarian primordia in *D. yakuba, D. simulans, D. mauritiana,* and *D. sechellia* are due to smaller embryonic or first larval instar ovarian primordia or to slower ovarian growth during the second larval instar, we counted the total numbers of cells at the onset of the second larval instar (Fig. 5A, 24 h after hatching). Only *D. sechellia* ovaries had significantly fewer cells than *D. melanogaster*-Sevelen at this stage (39 cells vs. 62 cells; $P = 0.007$, which is still lower than the tablewide level of significance, $P = 0.0071$; Rice 1989), suggesting that the smaller ovarian primordia in

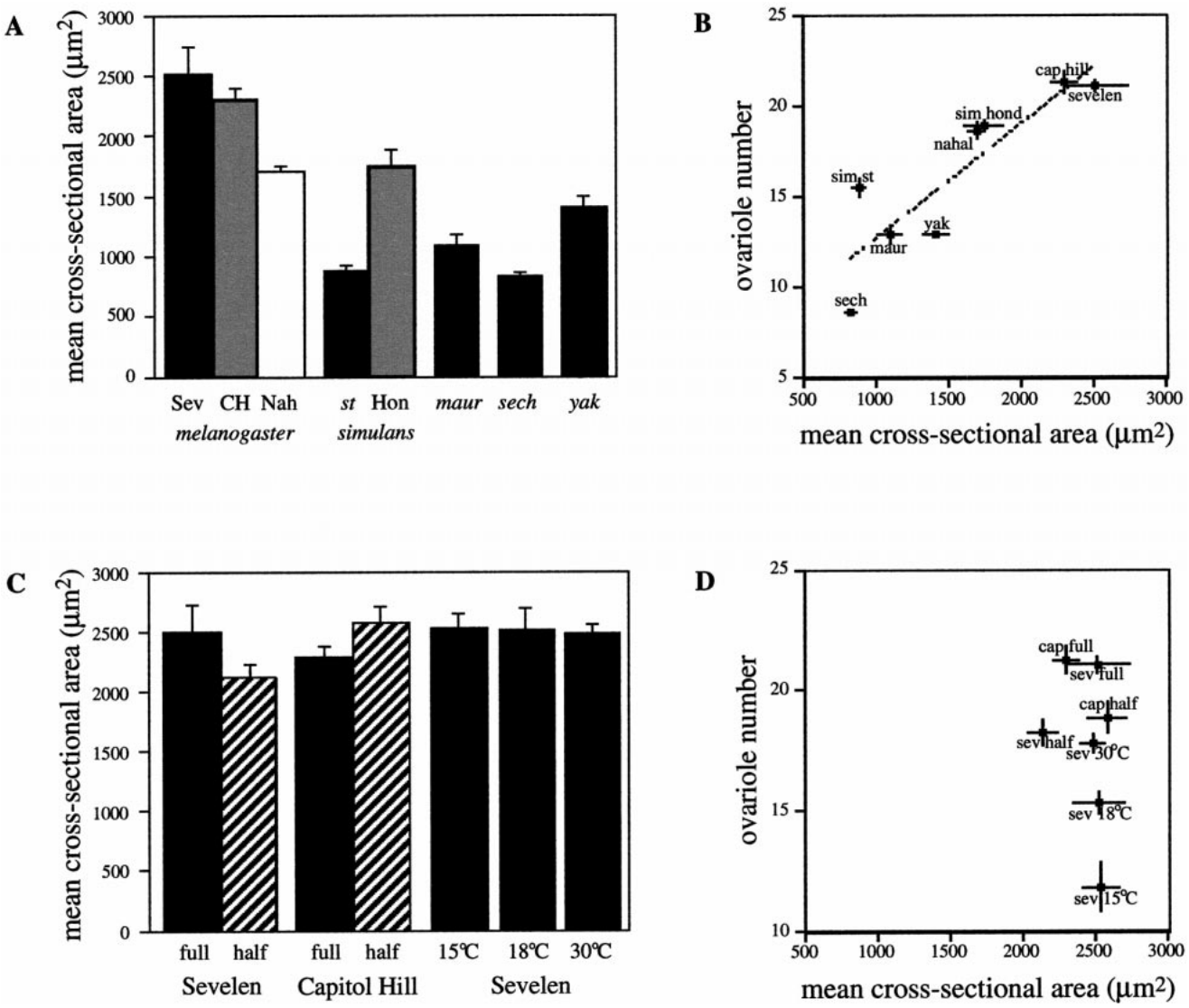

FIG. 4. Mean cross-sectional area of the immature ovary at the onset of the third larval instar (n = 10 ovaries, 10 animals): (A) in different species and populations; (B) as a function of adult ovariole number in different species and populations; (C) as a function of diet and temperature; and (D) as a function of adult ovariole number across different temperature and diet treatments. Abbreviations as in Figure 2A. All error bars are standard errors.

this species are due at least in part to differences arising during the embryonic or first larval stages. In contrast, Sevelen and *D. simulans-st* began the second instar with a similar number of cells in their ovaries (62 and 61 cells, respectively), but their cell numbers had diverged by the onset of the third larval instar (232 cells in Sevelen vs. 119 cells in *D. simulans-st*, $P < 0.001$; Fig. 5A, 24–48 h after hatching), resulting in significant differences in primordium size at the onset of the third instar. *Drosophila yakuba* ovaries were smaller than Sevelen ovaries at the onset of the second larval stage (50 cells vs. 62 cells), although not significantly so ($P = 0.07$, which is much higher than the tablewide level of significance: $P = 0.008$; Rice 1989). Similarly, the substantial difference in cell number between Sevelen and *D. mauritiana* at the onset of the third larval stage (232 cells vs. 177 cells; $P = 0.034$) is not significant at the tablewide level ($P = 0.0071$ in this case).

When the effects of body size (pupal weight) were removed, most of these differences in cell number during early stages were no longer significant. Still, it is clear that the growth rates (which are relative and, thus, independent of pupal weight) of the ovarian primordia in *D. simulans-st*, *D. mauritiana*, *D. sechellia*, and *D. yakuba* during the second instar were all substantially slower (more than 25% slower in all cases) than in *D. melanogaster*-Sevelen during the same period.

It is also possible that differences in mean cell size contributes to differences in ovarian primordium size. We could not accurately measure mean cell size in our experiments, because there is too much variability in cell sizes and shapes in immature *Drosophila* ovaries. Still, our indirect evidence (see Materials and Methods) suggests that there are substantial differences in ovarian cell size among the species examined (Fig. 5E). For example, at the onset of the third instar,

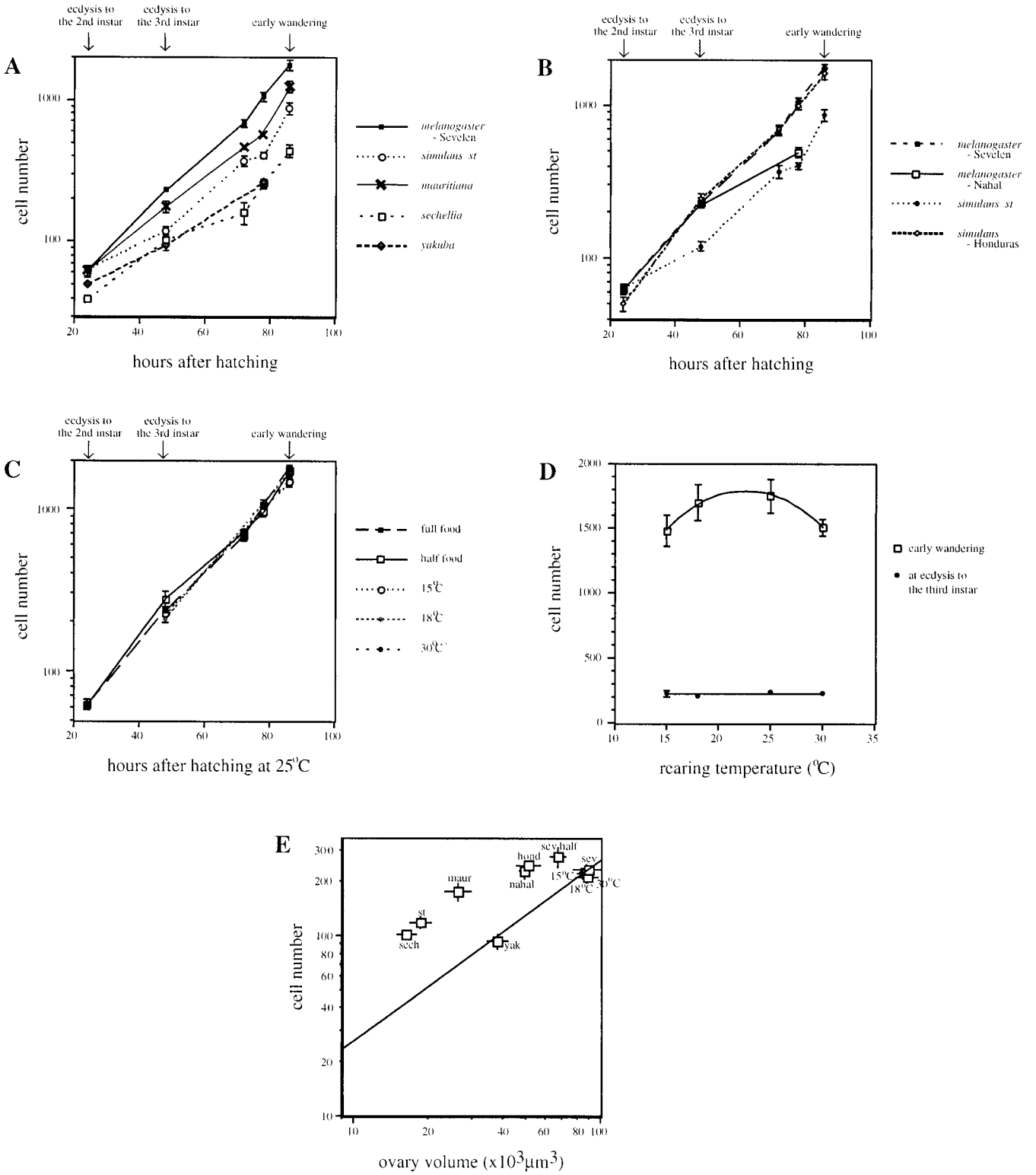

FIG. 5. Total ovarian cell numbers during the second and third larval stages (n = 5 ovaries, 5 animals). (A) Semi-log plot of interspecific variation in cell number. (B) Semi-log plot of interpopulational variation for the same trait. (C) Semi-log plot of cell numbers in *Drosophila melanogaster*-Sevelen under various environmental conditions. Full- and half-food larvae developed at 25°C. Times are hours after hatching at 25°C. For the 15°C, 18°C, and 30°C data, points are plotted according to the developmental stages noted at the top of the graph. (D) Linear plot of reaction norms for temperature-based plasticity at two larval stages. (E) Log-log plot of variability in cell size. Points lying along the line represent ovaries with cells of the same average size (volume) as in full food Sevelen larvae reared at 25°C (sev). Points lying above the line represent ovaries with smaller cells, on average, than in Sevelen ovaries; points lying below the line have larger cells. Distance from the line is an indication of the magnitude of these differences in average cell size. Abbreviations as in Figure 2. All error bars are standard errors.

the ovarian cells in *D. mauritiana, D. simulans-st*, and *D. sechellia* (which lie above the line in Fig. 5E; see legend) are, on average, approximately one-half the volume (not shown) of the cells at the same stage in *D. melanogaster*-Sevelen and *D. yakuba.* We have no evidence for phenotypic plasticity in cell number in *D. melanogaster*-Sevelen larvae reared at different temperatures (Fig. 5E), although it appears that half-fed Sevelen larvae have substantially smaller cells than do fully fed larvae (Fig. 5E; cell volume estimates not shown).

Growth in Third Instar Larval Ovaries

During the third larval instar, the ovaries of *D. melanogaster* grow almost 15-fold in volume (Kerkis 1931). To determine if differences in the growth rates of the ovaries during this period could account for differences in ovariole number, we counted total ovarian cell numbers at various times throughout the final (third) larval instar (Fig. 5A). If the growth rates in all of the species tested were similar, then the growth curves shown in Figure 5A would be essentially parallel between 48 h and 88 h after hatching, as we observed for *D. melanogaster*-Sevelen, *D. simulans-st*, and *D. mauritiana.* Sevelen third instar ovarian growth rates exceeded those of *D. simulans-st* and *D. mauritiana* by only 6% and 11%, respectively. In contrast, the third instar growth rate of Sevelen ovaries exceeded that of *D. sechellia* ovaries by 44%. The growth trajectory of *D. yakuba* ovaries was similar to that of *D. sechellia* (Fig. 5A). Consequently, the interspecific differences in ovarian size at the end of the third instar are due to differences in growth rates during the final two larval instars, with only *D. sechellia* and possibly *D. yakuba* showing a difference in ovarian growth rates in the embryo and/or first larval instar as well.

We also detected substantial interpopulational variation in ovarian growth trajectories (Fig. 5B). Whereas the ovaries of the Sevelen and Nahal strains of *D. melanogaster* began the third larval stage with similar cell numbers (232 cells vs. 230 cells; $P = 0.907$), they were significantly different 30 h later (1053 cells vs. 493 cells; $P = 0.002$). The two *D. simulans* strains, *st* and Honduras, also exhibited substantially divergent growth trajectories. In this case, the most profound differences arose during the second larval stage, where the Honduras growth rate exceeded that of the *st* strain by 153%.

We detected no difference in cell number between ovaries of *D. melanogaster*-Sevelen larvae reared under full- and half-food conditions at any stage (Fig. 5C; $P > 0.39$ at all stages examined). Rearing temperature also had no significant effect on cell number (Fig. 5C, D; $P > 0.095$ at all stages and temperatures). Although mean cell numbers at the early wandering stage differed slightly in different temperature treatments (Fig. 5D), none of these differences was significant ($P > 0.28$ in every pairwise case). Removing the effect of body size (pupal weight) had no qualitative effect on any of these results (not shown).

Therefore, whereas variations in ovarian growth trajectories correlate with interspecific and interpopulational ovariole number differences, there appears to be no role of growth trajectory differences in generating the substantial plasticity in ovariole number produced under extreme temperature and diet regimes.

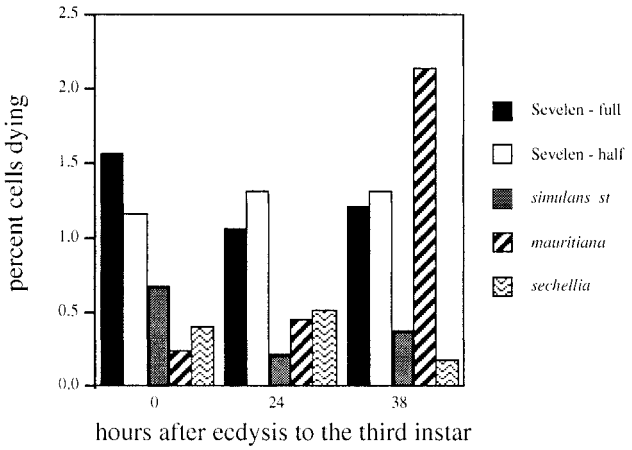

FIG. 6. Percent of cells undergoing cell death in third instar larval ovaries, as determined by the TUNEL method ($n = 10$ ovaries, 10 animals). Because the cell number and cell death data were obtained from different animals, statistical comparisons cannot properly be done here. However, there is substantial variation around these means for total numbers of dying cells (see Table 1). The values for percent dying cells are based on instantaneous counts at the time indicated.

Cell Death

During the final larval instar, the mean proportions of dying cells, as determined by the TUNEL method, in all of the ovaries examined were less than 2.5% (Fig. 6, Table 1). With only one exception, the proportion of dying cells in *D. melanogaster*-Sevelen ovaries was greater than in any of the other species tested This single exception (*D. mauritiana* at the early wandering stage, 38 h after ecdysis to the third instar), however, was probably not significant for two reasons: first, we found no evidence that dying cells were concentrated in the region of the ovary that gives rise to TF cells (not shown); and second, the growth rate of *D. mauritiana* ovaries during the wandering stage showed no signs of a decrease relative to that of Sevelen, suggesting that the increase in cell death was not producing a detectable effect on overall cell numbers (see Fig. 5A between 78 h and 88 h after hatching). We conclude that cell death during the larval stages plays essentially no role in generating the observed genetically fixed differences in ovariole number.

We observed no difference in ovarian cell death between full- and half-food *D. melanogaster*-Sevelen larvae (Fig. 6, Table 1), suggesting that cell death plays no role in ovariole number plasticity either.

To determine if cell death of fully formed TF stacks during metamorphosis might account for ovariole number differences, we compared the mean numbers of TF stacks formed at pupariation to the mean ovariole numbers in adults. We found no significant differences between TF number and adult ovariole numbers in any species or environmental regime (Table 2).

Therefore, unlike in honeybees (Hartfelder and Steinbrück 1997), we find no evidence that either genetically fixed or

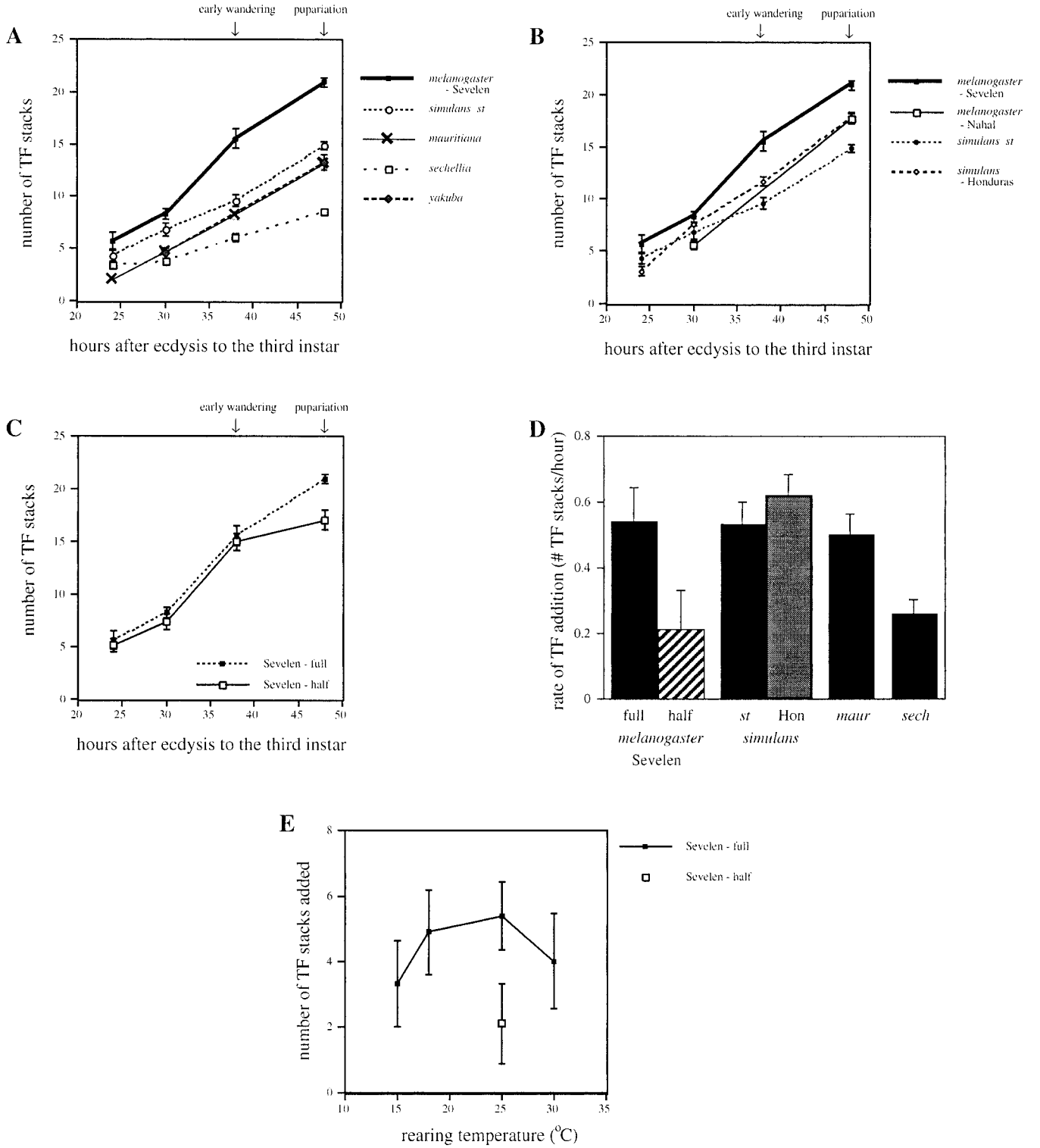

FIG. 7. Dynamics of terminal filament (TF) formation in the final larval stage (n = 10 ovaries, 10 animals): (A) interspecific variation in TF dynamics; (B) interpopulational variation in TF dynamics; (C) nutrient-based plasticity in TF dynamics in *Drosophila melanogaster*-Sevelen; (D) rate of TF addition during the wandering stage in different species and populations, and in half-food *D. melanogaster*-Sevelen. (abbreviations as in Fig. 2A); (E) norm of reaction for numbers of TF stacks added during the wandering stage as a function of temperature in *D. melanogaster*-Sevelen. All error bars are standard errors.

TABLE 1. Numbers of dying cells during the third larval instar. Values are means (± SE) of total numbers of TUNEL-labeled nuclei in 10 ovaries at each stage.

Hours after ecdysis to the third	*D. melanogaster* full food	*D. melanogaster* half food	*D. simulans-st*	*D. mauritiana*	*D. sechellia*
0	3.5 ± 1.0	3.2 ± 0.8	0.8 ± 0.3	0.4 ± 0.2	0.7 ± 0.4
24	7.2 ± 1.5	9.2 ± 1.9	0.8 ± 0.3	2.1 ± 0.6	0.8 ± 0.4
38	21.2 ± 3.6	22.4 ± 4.7	2.7 ± 0.9	26.0 ± 5.7	0.4 ± 0.3

plasticity-based differences in ovariole number are generated by increased proportions of dying cells in the ovaries of members of the *melanogaster* species group.

Dynamics of Terminal Filament Formation

The formation of TF stacks in *D. melanogaster* occurs over an approximately 36-h period at the end of larval life (King et al. 1968; Godt and Laski 1995; Hodin and Riddiford 1998). Differences in ovariole number may be due to differences in either the time of initiation or completion of TF formation or to changes in the rates at which TFs are added during this 36-h window.

Variability in the onset of terminal filament formation

In *D. melanogaster,* TFs begin to form between 12 h and 18 h after ecdysis to the third larval stage (Godt and Laski 1995; Hodin and Riddiford 1998), and by 24 h, all *D. melanogaster*-Sevelen ovaries have a few incipient TF stacks (J. Hodin, pers. obs.). At 24 hours there was no significant difference in mean TF number between *D. melanogaster*-Sevelen and *D. simulans-st* ($P = 0.743$; Fig. 7A). In contrast, *D. mauritiana* (mean = 2 TFs vs. 5.5 TFs; $P < 0.005$) had fewer TFs at this time than did Sevelen, indicating that there might be a slight delay in the onset of TF formation in this species. The difference between *D. sechellia* and Sevelen (mean = 3.3 TFs vs. 5.5 TFs; $P = 0.056$) is not significant at the tablewide level ($P = 0.017$; Rice 1989; Fig. 7A). Removing the effects of body size (pupal weight) had no qualitative effects on any of these results.

We also detected interpopulational variation in the onset of TF formation. The Honduras strain of *D. simulans* had significantly fewer TFs at 24 h when compared to the *D. simulans-st* strain (mean = 3.0 TFs vs. 4.2 TFs; $P = 0.004$; Fig. 7B), despite the fact that Honduras ultimately produces more ovarioles than *st* (19 vs. 16 ovarioles/ovary; $P < 0.001$; Fig. 2A).

At 24 h after ecdysis to the third instar, there was no difference between the mean numbers of TFs formed in full- and half-fed *D. melanogaster*-Sevelen (Fig. 7C; $P = 0.582$). Thus, we found no evidence for nutrient-based plasticity in the onset of TF formation in *D. melanogaster.*

Variability in the completion of terminal filament formation

In *D. melanogaster*, TF formation continues throughout the wandering stage until the time of pupariation, when the process of TF formation is complete (King et al. 1968; Hodin and Riddiford 1998; J. Hodin, pers. obs.). Similarly, in all of the other species studied, there is a substantial increase in TF number during the wandering stage (between 38 h and 48 h after ecdysis to the third instar, Fig. 7A, B, D), suggesting that TF formation continues at about the same rate throughout this period. In *D. sechellia,* the rate of TF formation during this stage was approximately twofold lower than any other species examined (Fig. 7D), but was not different than its rate throughout the third instar (Fig. 7A). Thus, early completion of TF formation apparently cannot account for differences in ovariole number in any of the species examined, although the formal possibility exists that TF formation may terminate early in *D. sechellia.*

Drosophila melanogaster-Sevelen raised under half-food conditions showed a substantial decrease in the rate of TF formation during the wandering stage (to less than half the

TABLE 2. Final mean terminal filaments (TFs) ± standard error (at pupariation) compared with adult mean ovariole numbers ± standard error.

Species/population	Mean TF number at pupariation[1]	Mean adult ovariole number[2]	*P*
melanogaster/Sevelen full food	20.9 ± 0.4	21.1 ± 0.3	0.47
melanogaster/Capitol Hill full food	20.1 ± 0.5	21.3 ± 0.6	0.24
melanogaster/Nahal	17.7 ± 0.6	18.6 ± 0.5	0.32
simulans/*st*	14.8 ± 0.4	15.5 ± 0.5	0.28
simulans/Honduras	17.9 ± 0.4	18.9 ± 0.3	0.12
mauritiana	12.8 ± 0.5	12.9 ± 0.5	0.94
sechellia	8.5 ± 0.2	8.5 ± 0.2	0.78
yakuba	13.8 ± 0.7	12.9 ± 0.2	0.20
melanogaster/Sevelen half food	17 ± 0.9	18.2 ± 0.5	0.36
melanogaster/Capitol Hill half food	17.2 ± 0.9	18.9 ± 0.6	0.08
melanogaster/Sevelen 15°C	11.9 ± 0.8	11.8 ± 1.0	0.95
melanogaster/Sevelen 18°C	17 ± 0.6	15.3 ± 0.5	0.07
melanogaster/Sevelen 30°C	18 ± 0.8	17.7 ± 0.4	0.77

[1] TF numbers are based on 10 ovaries dissected from 10 females, 0–2 h after pupariation.
[2] Adult ovariole numbers are based on dissections of 20 ovaries from 10 females.

TABLE 3. Mean terminal filaments (TFs) ± standard error, at early wandering and pupariation, of *D. melanogaster*-Sevelen larvae raised at various temperatures. TF numbers are based upon 10 ovaries dissected from 10 females at each stage.

	Rearing temperature (°C)			
Stage	15	18	25	30
Pupariation	11.9 ± 0.8	17.0 ± 0.6	20.9 ± 0.4	17.0 ± 0.8
Early wandering	8.6 ± 1.0	12.1 ± 1.2	15.5 ± 0.9	13.0 ± 1.2

rate as in full food Sevelen; Fig. 7C, D). To determine if this involved a cessation (rather than simply a decreased rate) of TF formation, we examined TF formation in late wandering animals, just a few hours before pupariation ($n = 6$). In several of these ovaries, TF formation was still progressing (data not shown), and no partially completed TF stacks were found in newly pupariated animals ($n = 25$; data not shown). Thus, the half-completed stacks at the late wandering stage presumably went on to complete TF formation by pupariation. We also examined the rates of TF accumulation during the wandering stage in Sevelen larvae raised on full food at 15°C, 18°C, and 30°C. Although there is some temperature-based plasticity in the rate of TF formation under these conditions, the differences are slight and not significant (Fig. 7E; see below).

Therefore, we find no evidence that the decreases in ovariole number are due to early cessation of TF formation, either in different species or in *D. melanogaster*-Sevelen larvae reared under different environmental conditions.

Variability in the rates of terminal filament formation

In *D. melanogaster*-Sevelen ovaries, TFs begin to form at a modest rate until 30 h after ecdysis to the third instar, at which point there is a sharp increase in the rate of TF formation. Thus, between 24 h and 30 h, the rate of TF addition in Sevelen was approximately 0.45 TF stacks per hour, whereas between 30 h and 38 h, the rate doubled to 0.9 TF stacks per hour (Fig. 7A). The other species failed to undergo this dramatic increase. For example, in *D. mauritiana,* the rate of TF addition only increased from 0.45 to 0.55 TF stacks per hour (Fig. 7A). We detected similar patterns in *D. simulans-st* and *D. yakuba* (although we only collected data at the 30 h and 48 h for the latter; Fig. 7A).

We also detected interpopulational differences in rates of TF formation (Fig. 7B). Between 24 h and 30 h, *D. simulans*-Honduras had the highest rate of TF formation among the species examined (greater than 0.7 TF stacks per hour), almost twice the rate seen in *D. simulans-st.* Apparently, it is this early spurt of TF formation that explains the difference in ovariole number between the two *D. simulans* populations. Although we only have data for 30 h and 48 h for the Nahal *D. melanogaster* strain, the rates of TF formation are similar to *D. melanogaster*-Sevelen during this interval (Fig. 7B).

Because larvae reared at different temperatures could only be accurately staged at wandering, we only have information on TF formation for the 15°C, 18°C, and 30°C Sevelen flies during the wandering stage (Fig. 7E). Due to differences in developmental rates, we express temperature-based TF differences as differences in total numbers of TF stacks added during the wandering stage. Fewer TFs were added at 15°C and 30°C, although the differences were not significant ($P > 0.3$; but see Materials and Methods). Importantly, the number of TF stacks added during the wandering stage was actually higher in 15°C Sevelen ovaries than in half-food Sevelen ovaries (3.3 TFs added vs. 2.1), despite the fact that half-food flies had more ovarioles (18 vs. 12; $P < 0.001$; Fig. 2). Second, at early wandering there were already substantial differences among TF numbers in the larvae raised at different temperatures (Table 3; although none of these differences were significant at the tablewide level; Rice 1989). Therefore, the temperature-induced ovariole number differences must have arisen earlier in the third instar.

DISCUSSION

There are two extreme views concerning the relationship between phenotypic plasticity and interspecific variation. In the first view, phenotypic plasticity provides the raw material for genetically fixed variation. If, for example, an extreme phenotype, such as white coloration, is produced under reduced temperature conditions, then the evolutionary fixation of the white color morph could occur if the developmental program induced by low temperature was activated constitutively. In the second view, phenotypic plasticity has no necessary direct connection to genetically fixed variation. To distinguish between these two disparate views, one must examine the underlying ontogenetic mechanisms that generate the different phenotypes. If the first view is correct, one would expect the developmental basis of the phenotypically plastic production of the white color morph to be the same as the developmental basis of the evolutionarily fixed white morphology. In the second view, the developmental bases could be different.

In this study, we set out to distinguish between these two alternate views by examining the mechanisms underlying the phenotypically plastic and genetically fixed variability in ovariole number in fruit flies of the *melanogaster* species group. We compared the reduction in ovariole number due to larval growth under different temperature and nutrient conditions to interspecific and interpopulational variability in ovariole number. *Drosophila melanogaster* larvae grown under reduced food conditions differ from fully fed *D. melanogaster* only in the rate of formation of the TF stacks (the larval precursors to the ovarioles) during the wandering stage at the end of larval life. At this stage, the larvae have completely assessed their nutritional state, and only then is TF formation affected. Temperature-induced plasticity in ovariole number is also due entirely to alterations in TF dynamics, but in this case, it may be due either to alterations in the initiation or in the rate of TF formation (or both) in the early to mid third larval instar and to slight alterations in the rate of TF formation during the wandering stage. These results are consistent with temperature-shift experiments showing

that the thermosensitive period for ovariole number in *D. melanogaster* covers the entire third larval instar (David et al. 1983). In sum, our data suggest that plasticity-based variability appears to be restricted to alterations in TF dynamics. Although we detected substantial variability among *melanogaster* populations in primordium size, this characteristic did not show significant plasticity-based variability within two *melanogaster* populations (see Fig. 4). It is important to note, however, that because we have examined the ontogenetic effects of environmental perturbations in only one species (*melanogaster*), and mainly in a single population (Sevelen), we cannot draw general conclusions regarding plastic responses within the *melanogaster* species group as a whole. Still, the data that we have on the plastic responses within *melanogaster* support our overall conclusions of limited plasticity-based variability in ontogenetic mechanisms.

Interspecific and interpopulational variation, by contrast, can have a wide range of underlying causes. For example, the smaller ovarian sizes in *D. simulans-st* and *D. mauritiana* (relative to *D. melanogaster*-Sevelen) are due almost entirely to slower ovarian growth rates during the second larval instar. In contrast, the differences in ovarian size between *D. sechellia* and *D. melanogaster*-Sevelen ovaries are due to slower ovarian growth rates in *D. sechellia* at earlier (embryonic or first instar) and later (third instar) stages. Similarly, interspecific and interpopulational variability in TF number is due mainly to delays in TF formation in some groups (such as *D. mauritiana* and the Honduras strain of *D. simulans*) and to slower rates of TF addition during the mid-third larval instar in others (such as in *D. simulans-st*).

Genetically fixed differences in body size (as measured by wing area) in *D. melanogaster* can be due either to differences in cell number or cell size (Partridge et al. 1994; de Moed et al. 1997). Our indirect evidence suggests that there are substantial differences in ovarian cell size among the species examined. We have no evidence for phenotypic plasticity in cell size or cell number in *D. melanogaster*-Sevelen larvae reared at different temperatures. In contrast, it appears that half-fed Sevelen larvae have substantially smaller cells than do fully fed larvae. These larvae then appear to compensate by producing slightly (although not significantly) more cells, resulting in immature ovaries of approximately the same size as in fully fed larvae.

Taken together, our results suggest that the scope of interspecific and interpopulational variability is greater than the scope of plasticity for the mechanisms underlying ovariole number differences within the *melanogaster* species group. Another way of viewing these results is that the processes of cell growth and cell division are dissociable from the processes of cell differentiation (namely, the formation of TF stacks) when the larvae are reared under differing environmental conditions. Perhaps it is the nature of a phenotypically plastic system that allows these different processes of growth and differentiation to be decoupled from one another, both phenotypically and genotypically. Furthermore, such dissociable developmental systems seem likely to be characterized by multiple, convergent mechanisms leading to similar phenotypic outcomes, because the underlying developmental mechanisms are not tightly integrated (Hodin 2000).

In sum, our data support the view that there is no direct connection between the mechanisms of plasticity and genetically fixed (i.e., interspecific and interpopulational) variation in this system. This does not necessarily suggest that plasticity and genetically fixed variation are entirely unrelated. Phenotypic plasticity, the production of alternative morphologies within a genotype, indicates that the underlying developmental mechanisms must be labile. In the case of the production of ovarioles, various environmental (such as food quality or temperature; Savilev 1928; Delpuech et al. 1995; Morin et al. 1996; Moreteau et al. 1997) and genetic (Garcia-Bellido 1963; Coyle-Thompson and Banerjee 1993; Lenz-Bohme et al. 1997; Hodin and Riddiford 1998) perturbations result in similar reductions in mean ovariole number. The terminal morphology (the production of functional ovarioles) appears to be stable in the face of these diverse perturbations. This observation is indicative of what Wagner and Misof (1993, p. 453) termed morphostatic constraints: ''Limitations to phenotypic variation caused by the stabilization of particular patterns rather than the inability of the generative processes of development to produce them in the first place.'' It seems that morphostatic constraint implies variability in developmental trajectories. Thus, the diverse perturbations (genetic and environmental) result in similar reductions in ovariole number, but by different developmental routes. Phenotypically plastic systems might be expected to exhibit genetically fixed variability in ''underlying generative processes'' simply because they are not constrained developmentally.

What is the genetic basis for interspecific and interpopulational fixed differences in ovariole number? Although numerous genes are known to affect ovarian morphogenesis and maturation (reviewed in King 1970; Spradling 1993), very few studies have reported ovariole numbers in flies carrying mutations for these genes. Reductions in ovariole numbers have been reported for mutations in the *furrowed* (Garcia-Bellido 1963), *Lamin* (Lenz-Bohme et al. 1997), and *strawberry notch* (Coyle-Thompson and Banerjee 1993) genes of *D. melanogaster*. We have shown previously (Hodin and Riddiford 1998) that mutations in the two genes whose protein products dimerize to form the functional ecdysone receptor, *Ecdysone Receptor* (*EcR*) and *ultraspiracle* (*usp*), result in decreases in ovariole number. Intriguingly, *EcR* heterozygotes and *EcR-usp* double heterozygotes show a delay in the onset of TF formation (Hodin and Riddiford 1998), a phenotype similar to that observed in this study for *D. mauritiana*, *D. sechellia*, and the Honduras strain of *D. simulans*. Wayne et al. (1997) used a quantitative genetic approach to demonstrate substantial genetic variation for ovariole number in *D. melanogaster*, confirming previous studies (Robertson 1957; Thomas-Orillard 1975; Boulétreau-Merle et al. 1982). Interestingly, Wayne and Mackay (1998) found that mutational heritability (V_M/V_E) for ovariole number in these lines was low (on the order of 10^{-4}), suggesting that a relatively small number of loci might contribute to ovariole number differences. In interspecific and interpopulational hybridization experiments, loci on chromosomes 2 and 3, but not on the X chromosome, had substantial effects on ovariole number, both within and between species in the *melanogaster* species group (Coyne et al. 1991; Chakir et al. 1995). It will

be interesting to see if, when, and where these genes are expressed during normal ovarian development. One might expect to find growth and cell division regulators, hormonally responsive genes, and regulators of TF differentiation among such loci.

Are there selective consequences to differences in ovariole number? The fact that there are reiterated latitudinal clines in ovariole number in *D. melanogaster* on several continents in both hemispheres (David and Bocquet 1975; Lemeunier et al. 1986; Capy et al. 1993; Azevedo et al. 1996) suggests that ovariole number responds to selection. Alternately, differences in ovariole number may be a correlated response to selection on other latitudinally responsive features. Still, ovariole number has been shown to correlate with egg-laying capacity, and thus, fitness (David 1970; Cohet and David 1978; Boulétreau-Merle et al. 1982). If this is the case, is there any advantage to having a *reduced* ovariole number? There may be costs associated with the large ovaries indicative of flies with high ovariole numbers, including costs to flight maneuverability (Berrigan 1991) or developmental production costs (trade-offs sensu Nijhout and Emlen 1998). The general trend in Hawaiian drosophilids is that fly species that tend to oviposit a few eggs at a time on leaves have low ovariole numbers, whereas those that oviposit multiple eggs at once in stems or under bark have higher ovariole numbers (Kambysellis and Heed 1971; Kambysellis et al. 1995). These results suggest that low ovariole numbers may be favored in insects that lay fewer eggs per day and raise the possibility that there is a selective advantage to the low ovariole numbers in *D. sechellia* (R'Kha et al. 1997). These flies avoid interspecific competition by ovipositing in *M. citrifolia,* a fruit that is toxic to other sympatric drosophilids (Louis and David 1986; R'Kha et al. 1991; Legal et al. 1994; Farine et al. 1996; R'Kha et al. 1997; Amlou et al. 1998).

Ovariole numbers show marked interspecific as well as interpopulational variability. Therefore, reconstructing ancestral character states with respect to ovariole number is at best a questionable enterprise. With this caveat in mind, the data presented here suggest several trends in evolutionary changes in ovarian morphogenesis and ovariole number within the *melanogaster* species group (Fig. 1, phylogeny with mapped character states). For example, it appears that there has been an overall increase in ovariole number in *D. melanogaster,* which may have been facilitated by an apparent increase in the size of the ovarian primordium in this species. It will be interesting to see if other drosophilids with large ovariole numbers (Mahowald and Kambysellis 1980) also have evolved larger ovarian primordia. Such patterns would suggest that primordium size might be a developmental constraint on the maximum obtainable ovariole number in drosophilids. It is also interesting to note that for several characters, most notably for the cell number growth trajectories, *D. sechellia,* the most derived species in the group, and *D. yakuba,* our outgroup, are more similar to one another than they are to any of the other species examined. Again, the fact that these characters are evolving even within species suggests that any ancestral state reconstructions for such characters would be little more than guesswork.

Now that we have begun to define the developmental bases for (both plastic and genetically fixed) ovariole number differences, it will be possible to determine the genetic bases for such developmental differences. How easy or difficult is it to evolve such changes? Do independent instances of evolutionary changes in ovariole number with similar developmental bases have similar or dissimilar genetic bases? Such information would combine macroevolution with population genetics and developmental biology, thus bringing us one step closer to understanding the mechanistic basis of evolutionary change.

Acknowledgements

We thank J. Kingsolver, J. Truman, R. Huey, and two anonymous reviewers for comments on this manuscript. We are grateful to M. Schubiger for helping us devise the cell-counting protocol, and to R. Ricker and S. Bellman for expert cell-counting assistance. We also thank G. Schubiger for the use of his *D. melanogaster*-Sevelen flies and various equipment; B. Wakimoto for the *D. mauritiana, D. simulans-st* and *D. yakuba* flies; the Bloomington Species Center for sending us the *D. sechellia, D. simulans*-Honduras, and Peru flies; A. Korol for the *D. melanogaster*-Nahal flies; T. Bilgen for translating a French article; J. Neuharth in the fly kitchen for food preparation; J. Kingsolver for use of his incubator; G. Odell and G. von Dassow for help at key moments; F. Kafatos for the USP antibody; and A. Hoang, E. Meir, R. Huey, and D. Berrigan for help with statistics. This work was supported by National Institutes of Health grant NS29971 (to J. W. Truman and LMR). JH was supported by National Institutes of Health training grant HD07183 (to JH).

Literature Cited

Amlou, M., B. Moreteau, and J. R. David. 1998. Larval tolerance in the *Drosophila melanogaster* species complex toward the two toxic acids of the *D. sechellia* host plant. Hereditas 129:7–14.

Azevedo, R. B. R., V. French, and L. Partridge. 1996. Thermal evolution of egg size in *Drosophila melanogaster.* Evolution 50: 2338–2345.

Berrigan, D. 1991. Lift production in the flesh fly, *Neobellieria* (= *Sarcophaga*) *bullata* Parker. Funct. Ecol. 5:448–456.

Boulétreau-Merle, J., R. Allemand, Y. Cohet, and J. R. David. 1982. Reproductive strategy in *Drosophila melanogaster*: significance of genetic divergence between temperate and tropical populations. Oecologia 53:323–329.

Brakefield, P. M., J. Gates, D. Keys, F. Kesbeke, P. J. Wijngaarden, A. Monteiro, V. French, and S. B. Carroll. 1996. Development, plasticity and evolution of butterfly eyespot patterns. Nature 384: 236–242.

Brakefield, P. M., F. Kesbeke, and P. B. Koch. 1998. The regulation of phenotypic plasticity of eyespots in the butterfly *Bicyclus anynana.* Am. Nat. 152:853–860.

Caccone, A., E. N. Moriyama, J. M. Gleason, L. Nigro, and J. R. Powell. 1996. A molecular phylogeny for the *Drosophila melanogaster* subgroup and the problem of polymorphism data. Mol. Biol. Evol. 13:1224–1232.

Capy, P., E. Pla, and J. R. David. 1993. Phenotypic and genetic variability of morphometrical traits in natural populations of *Drosophila melanogaster* and *D. simulans.* I. Geographic variations. Genet. Sel. Evol. 25:517–536.

Carlson, K. A., T. J. Nusbaum, M. R. Rose, and L. G. Harshman. 1998. Oocyte maturation and ovariole number in lines of *Drosophila melanogaster* selected for postponed senescence. Funct. Ecol. 12:514–520.

Chakir, M., J. R. David, E. Pla, and P. Capy. 1995. Genetic basis

of some morphological differences between temperate and equatorial populations of *Drosophila melanogaster*. Experientia 51: 744–748.
Chippindale, A. K., A. M. Leroi, S. B. Kim, and M. R. Rose. 1993. Phenotypic plasticity and selection in *Drosophila* life-history evolution. 1. Nutrition and the cost of reproduction. J. Evol. Biol. 6:171–193.
Cochran, W. G. 1957. Analysis of covariance: its nature and uses. Biometrics 13:261–281.
Cohet, Y., and J. David. 1978. Control of the adult reproductive potential by preimaginal thermal conditions: a study in *Drosophila melanogaster*. Oecologia 36:295–306.
Cox, D. R. 1958. Planning of experiments. Wiley, New York.
Coyle-Thompson, C. A., and U. Banerjee. 1993. The *strawberry notch* gene functions with *Notch* in common developmental pathways. Development 119:377–395.
Coyne, J. A., J. Rux, and J. R. David. 1991. Genetics of morphological differences and hybrid sterility between *Drosophila sechellia* and its relatives. Genet. Res., Camb. 57:113–122.
David, J. R. 1970. Le nombre d'ovarioles chez *Drosophila melanogaster*: relation avec la fécondité et valeur adaptative. Arch. Zool. Exp. Gén. 111:357–370.
David, J. R., and C. Bocquet. 1975. Evolution in a cosmopolitan species: genetic latitudinal clines in *Drosophila melanogaster* wild populations. Experientia 31:164–166.
David, J. R., R. Allemand, J. Van Herrewege, and Y. Cohet. 1983. Ecophysiology: abiotic factors. Pp. 105–170 *in* M. Ashburner, H. L. Carson, and J. N. Thompson Jr., eds. The genetics and biology of *Drosophila*. Vol. 3D. Academic Press, San Francisco, CA.
Delpuech, J.-M., B. Moreteau, J. Chiche, E. Pla, J. Vouidibio, and J. R. David. 1995. Phenotypic plasticity and reaction norms in temperate and tropical populations of *Drosophila melanogaster*: ovarian size and developmental temperature. Evolution 49: 670–675.
de Moed, G. H., G. de Jong, and W. Scharloo. 1997. The phenotypic plasticity of wing size in *Drosophila melanogaster*: the cellular basis of its genetic variation. Heredity 79:260–267.
Farine J. P., L. Legal, B. Moreteau, and J. L. LeQuere. 1996. Volatile components of ripe fruits of *Morinda citrifolia* and their effects on *Drosophila*. Phytochemistry 41:433–438.
Finney, D. J. 1957. Stratification, balance, and covariance. Biometrics 13:373–386.
Garcia-Bellido, A. 1963. Fenogenetica del locus *furrowed* (*fw*) de *Drosophila melanogaster*. Genetica Iberica 15:1–74.
Godt, D., and F. A. Laski. 1995. Mechanisms of cell rearrangement and cell recruitment in *Drosophila* ovary morphogenesis and the requirement of *bric à brac*. Development 121:173–187.
Hartfelder, K., and G. Steinbrück. 1997. Germ cell cluster formation and cell death are alternatives in caste-specific differentiation of the larval honey bee ovary. Invert. Reprod. Dev. 31:237–250.
Hodin, J. 2000. Plasticity and constraints in development and evolution. J. Exp. Zool. 288:1–20.
Hodin, J., and L. M. Riddiford. 1998. The ecdysone receptor and ultraspiracle regulate the timing and progression of ovarian morphogenesis during *Drosophila* metamorphosis. Dev. Genes Evol. 208:304–317.
Huey, R. B., T. Wakefield, W. D. Crill, and G. W. Gilchrist. 1995. Within-generation and between-generation effects of temperature on early fecundity of *Drosophila melanogaster*. Heredity 74: 216–223.
Kambysellis, M. P., and W. B. Heed. 1971. Studies of oogenesis in natural populations of Drosophilidae. I. Relation of ovarian development and ecological habitats of the Hawaiian species. Am. Nat. 105:31–49.
Kambysellis, M. P., K.-F. Ho, E. M. Craddock, F. Piano, M. Parisi, and J. Cohen. 1995. Pattern of ecological shifts in the diversification of Hawaiian *Drosophila* inferred from a molecular phylogeny. Curr. Biol. 5:1129–1139.
Kerkis, J. 1931. The growth of the gonads in *Drosophila melanogaster*. Genetics 16:212–224.
———. 1933. Development of gonads in hybrids between *Drosophila melanogaster* and *Drosophila simulans*. J. Exp. Zool. 66: 477–509.
Khoury-Christianson, A. M., D. L. King, E. Hatzivassiliou, J. E. Casas, P. L. Hallenbeck, V. M. Nikodem, S. A. Mitsialis, and F. C. Kafatos. 1992. DNA binding and heterodimerization of the *Drosophila* transcription factor chorion factor 1/*ultraspiracle*. Proc. Natl. Acad. Sci. USA 89:11503–11507.
King, R. C. 1970. Ovarian development in *Drosophila melanogaster*. Academic Press, New York.
King, R. C., S. K. Aggarwal, and U. Aggarwal. 1968. The development of the female *Drosophila* reproductive system. J. Morph. 124:143–166.
Lachaise, D., M.-L. Cariou, J. R. David, F. Lemeunier, L. Tsacas, and M. Ashburner. 1988. Historical biogeography of the *Drosophila melanogaster* species subgroup. Evol. Biol. 22:159–226.
Legal, L., B. Chappe, and J. M. Jallon. 1994. Molecular basis of *Morinda citrifolia* (L) toxicity on *Drosophila*. J. Chem. Ecol. 20: 1931–1943.
Lemeunier, F., J. R. David, L. Tsacas, and M. Ashburner. 1986. Geographic variations in climatic adaptations. Pp. 147–256 *in* M. Ashburner, ed. The genetics and biology of *Drosophila*. Vol. 3E. Academic Press, San Francisco, CA.
Lenz-Bohme, B., J. Wismar, S. Fuchs, R. Reifegerste, E. Buchner, H. Betz, and B. Schmitt. 1997. Insertional mutation of the *Drosophila* nuclear lamin Dm_0 gene results in defective nuclear envelopes, clustering of nuclear pore complexes, and accumulation of annulate lamellae. J. Cell Biol. 137:1001–1016.
Louis, J., and J. R. David. 1986. Ecological specialization in the *Drosophila melanogaster* species subgroup: a case study of *D. sechellia*. Acta Oecol., Oecol. Gen. 7:215–229.
Mahowald, A. P., and M. P. Kambysellis. 1980. Oogenesis. Pp. 141–224 *in* M. Ashburner and T. R. F. Wright, eds. The genetics and biology of *Drosophila*. Vol. 2D. Academic Press, London.
Moreteau, B., J.-P. Morin, P. Gibert, G. Pétavy, E. Pla, and J. R. David. 1997. Evolutionary changes in nonlinear reaction norms according to thermal adaptation: a comparison of the *Drosophila* species. C. R. Acad. Sci. Paris, Sciences de la Vie 320:833–841.
Morin, J.-P., B. Moreteau, G. Pétavy, R. Parkash, and J. R. David. 1997. Reaction norms of morphological traits in *Drosophila*: adaptive shape changes in a stenotherm circumtropical species? Evolution 51:1140–1148.
Nijhout, H. F., and D. J. Emlen. 1998. Competition among body parts in the development and evolution of insect morphology. Proc. Natl. Acad. Sci. USA 95:3685–3689.
Pappas, N., and L. Engstrom. 1974. Survey of ovariole numbers in wild-type *Drosophila melanogaster*. Drosophila Inf. Serv. 51:27.
Partridge, L., B. Barrie, K. Fowler, and V. French. 1994. Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. Evolution 48:1269–1276.
Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution 43:223–225.
R'kha, S., P. Capy, and J. R. David. 1991. Host plant specialization in the *Drosophila melanogaster* species complex A: physiological, behavioral, and genetic analysis. Proc. Natl. Acad. Sci. USA 88:1835–1839.
R'kha, S., B. Moreteau, J. A. Coyne, and J. R. David. 1997. Evolution of a lesser fitness trait: egg production in the specialist *Drosophila sechellia*. Genet. Res. 69:17–23.
Robertson, F. W. 1957. Studies in quantitative inheritance. X. Genetic variation of ovary size in *Drosophila*. J. Genet. 55:410–427.
Rountree, D. B., and H. F. Nijhout. 1995a. Hormonal control of a seasonal polyphenism in *Precis coenia* (Lepidoptera: Nymphalidae). J. Insect Physiol. 41:987–992.
———. 1995b. Genetic control of a seasonal morph in *Precis coenia* (Lepidoptera: Nymphalidae). J. Insect Physiol. 41:1141–1145.

Ruttner, F. 1988. Biogeography and taxonomy of honeybees. Springer Verlag, New York.

Saviliev, V. I. 1928. Manifold effects of the gene *vestigial* in *Drosophila melanogaster*. Trud. Leningr. Obst. Estestv. 58:65–88.

Schlichting, C. D., and M. Pigliucci. 1998. Phenotypic evolution: a reaction norm perspective. Sinauer, Sunderland, MA.

Sokal, R. R., and F. J. Rohlf. 1981. Biometry. 2d ed. W. H. Freeman, New York.

Spradling, A. 1993. Developmental genetics of oogenesis. Pp. 1–70 *in* M. Bate and A. Martinez-Arias, eds. The development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Plainview, NY.

Thomas-Orillard, M. 1975. Tentative localization of genes important in determining the number of ovarioles in *Drosophila*. Arch. Genet. 48:116–127.

Wagner, G. P., and B. Y. Misof. 1993. How can a character be developmentally constrained despite variation in developmental pathways? J. Evol. Biol. 6:449–455.

Wayne, M. L., and T. F. C. Mackay. 1998. Quantitative genetics of ovariole number in *Drosophila melanogaster*. II. Mutational variation and genotype-environment interaction. Genetics 148: 201–210.

Wayne, M. L., J. B. Hackett, and T. F. C. Mackay. 1997. Quantitative genetics of ovariole number in *Drosophila melanogaster*. I. Segregating variation and fitness. Evolution 51:1156–1163.

Williamson, A., and R. Lehmann. 1996. Germ cell development in *Drosophila*. Ann. Rev. Cell Dev. Biol. 12:365–391.

Zander, E., F. Lüschel, and K. Meier. 1916. Die Ausbildung des Geschlechts bei der Honigbiene (*Apis mellifera* L.). Z. Angew. Entomol. 3:1–74.

Zar, J. H. 1984. Biostatistical analysis. 2d ed. Prentice Hall, Toronto.

Corresponding Editor: W. T. Starmer