

ENVIRONMENTAL HETEROGENEITY AND THE MAINTENANCE OF GENETIC VARIATION FOR REPRODUCTIVE DIAPAUSE IN *DROSOPHILA MELANOGASTER*

PAUL S. SCHMIDT¹ AND DAPHNE R. CONDE

Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

¹E-mail: schmidtp@sas.upenn.edu

Abstract.—*Drosophila melanogaster* has colonized temperate habitats on multiple continents over a historical time period, and many traits vary predictably with latitude. Despite considerable attention paid to clinal variation in *Drosophila*, the mechanisms generating such patterns in nature remain largely unidentified. In *D. melanogaster*, the expression of reproductive diapause can be induced by exposure to low temperatures and shortened photoperiods. Both diapause expression itself and the underlying genetic variance for diapause expression have widespread impacts on organismal fitness, and diapause incidence exhibits a 60% cline in frequency in the eastern United States. The major aim of this study was to evaluate whether the relative fitness of diapause and nondiapause genotypes varies predictably with environment. In experimental population cages in the laboratory, the frequency of genotypes that express diapause increased over time when flies were exposed to environmental stress, whereas the frequency of nondiapause genotypes increased when flies were cultured under benign control conditions. Other fitness traits correlated with the genetic variance for diapause expression (longevity, mortality rates, stress resistance, lipid content, preadult viability, fecundity profiles, and development time) also diverged between experimental treatments. Similarly, sampling of isofemale lines from natural populations revealed that the frequency of diapause incidence cycled over time in seasonal habitats: diapause expression was at high frequency following the winter season and subsequently declined throughout the summer months. In contrast, diapause expression was low and temporally homogeneous in isofemale line collections from human-associated urban habitats. These data suggest that genetic variation underlying the diapause-nondiapause dichotomy may be actively maintained by selection pressures that vary spatially and temporally in natural populations.

Key words.—Clines, diapause, *Drosophila*, life-history trade-offs.

Received July 27, 2005. Accepted June 12, 2006.

Understanding the mechanisms by which organisms adapt to environmental heterogeneity remains a fundamental goal in evolutionary biology. Clines have long been used to infer the action of natural selection on particular traits or polymorphisms across environmental gradients (e.g., Endler 1986). The inference that a specific cline is generated by selection can be indirectly evaluated by several methods, including examining patterns of variation at multiple loci (e.g., Berry and Kreitman 1993; Sezgin et al. 2004), among different taxa (Karan et al. 1998), and among replicate samples (e.g., Oakeshott et al. 1982; Gockel et al. 2001). However, the null hypothesis of selective neutrality of molecular or phenotypic variants must be directly tested by experimentation. Identifying the mechanisms that generate clines, even for well-studied polymorphisms in model organisms, often proves difficult. In *Drosophila melanogaster*, a number of traits and allele frequencies at specific loci vary significantly with latitudinal origin of populations on multiple continents (e.g., Boulétreau-Merle et al. 1982; Hoffmann et al. 2001, 2002; Sezgin et al. 2004). While geographic variation in reproductive and stress-related traits may reflect adaptation to novel temperate environments (Mitrovski and Hoffmann 2001; Boulétreau-Merle and Fouillet 2002; Hoffmann et al. 2003), the selective dynamics that generate these clines in natural populations remain largely unresolved.

Reproductive or ovarian diapause in *D. melanogaster* is an ideal model system for an experimental, mechanistic dissection of a cline. The diapause syndrome, an intensively characterized adaptation to seasonality in insects, is defined as a neuroendocrine-mediated genetic program elicited by token environmental cues such as photoperiod and/or temperature (Tauber et al. 1986). Expression of the syndrome is com-

monly associated with reproductive quiescence and a variety of physiological changes, such as delayed senescence, elevated stress resistance, and depressed metabolism, that promote survivorship over prolonged periods of unfavorable conditions (e.g., winter). The expression of diapause is highly species specific; in the Drosophilidae, diapause is most commonly expressed in adults (Lumme and Lakovaara 1983).

Ovarian or reproductive diapause in *D. melanogaster* was first described by Saunders et al. (1989), and patterns of diapause expression demonstrate some similarities to well-characterized diapause syndromes of other insect taxa. First, diapause is induced by exposure to shortened photoperiod (<12 h light) and low temperature ($\leq 12^{\circ}\text{C}$), token environmental cues routinely used for time measurement in arthropods. *Drosophila melanogaster* females measure night rather than day length (Saunders 1990). However, the relative importance of photoperiod versus temperature is unclear: in Canton-S females, both photoperiod and temperature impact diapause expression (Saunders et al. 1989), whereas no widespread effects of photoperiod are evident in other lines more recently sampled from natural populations (Tatar et al. 2001). Second, diapause expression appears to be modulated by neuroendocrine signaling, as exogenous application of juvenile hormone analogs terminates diapause, and diapause expression is associated with a reduced rate of juvenile hormone synthesis in the corpora allata (Saunders et al. 1990). Studies by Richard et al. (1998, 2001) also implicated a role for ecdysteroids in mediating vitellogenesis and the diapause response and further supported that *D. melanogaster* diapause is under neuroendocrine control. Third, the potential fitness advantages of diapause expression in *D. melanogaster* have been described by Tatar et al. (2001): exposure to diapause-

inducing conditions for both males and females results in life-span extension, negligible senescence while in diapause, and increased resistance to multiple forms of environmental stress. Finally, patterns of diapause expression vary predictably with latitude in *D. melanogaster* (Williams and Sokolowski 1993; Schmidt et al. 2005a), as they do in many other species (reviewed in Danilevskii 1965; Tauber et al. 1986).

Although the reproductive diapause of *D. melanogaster* has not been extensively characterized, several observations suggest that the phenotype may be qualitatively distinct from a true diapause syndrome. Most notably, diapause is terminated almost immediately upon transfer of females to warmer temperatures and/or longer photoperiods, rather than being of a predetermined duration. The observation that diapause is both initiated and terminated directly by environmental variables suggests a facultative quiescence or oligopause (Saunders et al. 1989). Furthermore, the expression of diapause is highly variable within natural populations: when exposed to the standard diapause assay (Saunders et al. 1989; Williams and Sokolowski 1993; Tatar et al. 2001), some strains remain reproductively quiescent (diapause genotypes), whereas others progress through normal reproductive development (nondiapause genotypes). Patterns of inheritance for diapause in *D. melanogaster* appear relatively straightforward (Williams and Sokolowski 1993; Schmidt et al. 2005a). Saunders and Gilbert (1990) suggested that the reproductive quiescence of *D. melanogaster* may be of recent evolutionary origin. Consistent with this hypothesis, diapause expression in the standard assay has not been observed in African populations of *D. melanogaster* or in natural populations of *D. simulans* from North America (P. Schmidt, unpubl. data).

In the eastern United States, diapause incidence exhibited a 60% cline in frequency among isofemale line populations sampled from 11 different latitudes (Schmidt et al. 2005a); molecular variants thought to be neutral with respect to fitness did not demonstrate any such patterns of geographic variation in these same sampled populations (Schmidt et al. 2000; Sezgin et al. 2004). Such a pattern is consistent with selection on diapause expression and overwintering in temperate habitats, or it may represent an indirect effect of selection on correlated traits. Analyses of diapause and nondiapause inbred lines indicated that the cline in diapause incidence may reflect a series of life-history trade-offs associated with the genetic variance for diapause expression (Schmidt et al. 2005b). In the absence of cues that would elicit diapause expression, diapause genotypes were constitutively longer lived and more resistant to cold and starvation stress than were nondiapause lines. However, nondiapause genotypes were also characterized by higher early-life fecundity and a faster rate of development from egg to adult.

The life-history trade-off hypothesis predicts that the relative fitness of diapause and nondiapause genotypes is dependent on specific environmental parameters that vary with latitude and/or season. Here, we provide an initial test of this prediction by competing diapause and nondiapause genotypes under two environmental regimes in experimental population cages in the laboratory. Seasonal collections were also made from natural populations to evaluate temporal change in the frequency of diapause genotypes in two distinct environments.

MATERIALS AND METHODS

Experimental Setup

Isofemale lines were collected from Walpole, Maine, in September 2001. Each line was subsequently cultured at 25°C and a 12:12 L:D photoperiod, inbred by 25 generations of full-sib mating, and characterized for diapause phenotype (Saunders et al. 1989). Of the sets of diapause (D) and nondiapause (ND) lines previously described (Schmidt et al. 2005b), 10 lines of each genotype were randomly selected for the experiments described here. Because we expected that there would be substantial variation among inbred lines within each genotypic class (D and ND), the experimental setup incorporated replication at the level of genetic background. Of the 10 replicate lines for each genotype, five were randomly selected and assigned to genetic background A (D: CC25, CC93, CC143, RR11, RR43; ND: CC21, CC42, CC79, RR42, RR44). The remaining lines were assigned to genetic background B (D: CC37, CC39, CC120, RR8, RR24; ND: CC44, CC109, CC146, RR12, RR90). The A and B population cages were initiated with the same D:ND genotypic ratio, but were comprised of distinct lines of independent genetic backgrounds. Prior to the initiation of the experimental population cages, each of the 10 selected lines was maintained in four replicate cultures in 175-ml bottles at relatively constant density (200 ± 20 eggs/bottle) for two generations. Fifty flies of each sex were collected as virgins within a 2-h window across all replicates for each line. Ten flies of each sex from each line were then used to establish four replicate experimental population cages (1450BSV, BioQuip Products, Gardena, CA) for each genetic background. All cages were initiated with 10 distinct lines (five D and five ND lines per cage) \times 10 flies per sex for a total of 200 flies in each. Within the A and B genetic backgrounds, two cages were randomly assigned to the stress treatment and two cages to the control treatment. Thus, the experiment consisted of a total of eight experimental population cages: four cages each for the independent A and B backgrounds, with half of the cages being assigned to each experimental treatment (stress and control). All cages were maintained with a generation time of 14 days, and the experiment was run for a total of 13 generations.

After establishment, cages were kept under control conditions (25°C, 12:12 L:D, constant humidity) on banana-molasses medium for two generations. Flies in the stress treatment were then exposed to alternating bouts of starvation and cold stress. Starvation stress was simulated by removing food for 3 days; cold stress consisted of exposure to -20°C for 45 min. These treatments were used as token stressors following the methodology of Schmidt et al. (2005b) and were not designed to accurately simulate ecologically relevant parameters. Following each round of stress exposure, cages were kept under control conditions for one generation such that the density of adults remained relatively constant across treatments. In the control treatment, flies were kept at 25°C, 12:12 L:D, and constant humidity for the duration of the experiment.

Diapause Phenotyping: Cages

The percentage of females that expressed reproductive diapause in the eight cages was estimated at five time points: in the F_1 generation and in the generations following each round of stress exposure. From all cages, approximately 100 females were collected within 2 h posteclosion and placed in groups of 10 in fresh vials at 11°C and 10:14 L:D. Ovaries were dissected 4 weeks later and characterized according to King (1970). A fly was scored as diapausing if the most advanced stage oocyte was \leq stage 7. Data were analyzed with nominal logistic regression in JMP version 5 (SAS Institute, Cary, NC), modeling the log odds of diapause/non-diapause.

Life-History Trait Measurements

Following cage establishment, patterns of longevity and fecundity were determined in the second generation (prior to stress exposure) to evaluate potential cage and background effects. From each cage, 20 virgins of each sex were randomly collected within a 4-h window and placed in 20 separate vials (one male and one female in each). Additional collections were made at the same time for replacement of dead males and females. Each vial was transferred daily and the number of eggs counted until both original individuals had died. These estimates of longevity and patterns of reproduction were repeated at the end of the experiment, subsequent to all rounds of stress exposure. Mortality data were analyzed with a proportional hazards model using JMP version 5.

The final analyses for all assayed traits (life span, fecundity, development time, viability, lipid content, dry weight) were conducted on flies that had been passed through two generations of low-density culture. In the 11th generation of culture, following the last stress exposure and subsequent recovery generation, 10 fresh egg-collection plates (thinly poured banana-molasses medium) were placed in each of the eight cages. Flies were allowed to oviposit for 24 h, after which time groups of eggs were placed in 20 replicate 175-ml culture bottles at a density of 200 ± 20 eggs/bottle. After eclosion in this first generation of low-density culture, flies were transferred to new bottles, allowed to oviposit, and density standardized as before. Virgin males and females were then collected in 4-h cohorts over 12 h and used as experimental material for all final analyses. Life span, fecundity, lipid content, development time, and viability were assayed simultaneously on flies collected in the 13th generation following cage initiation. Thus, the reported trait differences between treatments reflect genetic change over generational time and not trait plasticity in response to varying levels of stress exposure.

Total body lipid content was measured by ether extraction upon experimental termination according to the methodology of Robinson et al. (2000). Approximately 100 flies of each sex were collected from bottles originating from each cage, separated into mixed sex groups of 10, and aged to 5 days. Flies were then separated by sex into groups of 10 in microcentrifuge tubes and frozen at -80°C until analysis. An initial regression of lipid content on dry mass was performed; the resulting residuals were analyzed with ANOVA. All analyses of variance were conducted in JMP version 5.

Development time and egg-to-adult viability were also measured for all cages subsequent to experimental manipulation. Twenty virgin males and females were collected from bottles originating from each cage and placed in mixed sex groups of 10 in fresh vials. After 5 days of exposure to mates, flies were transferred to new vials and allowed to oviposit for 1 h. The total number of eggs laid was then counted and standardized at 30 eggs/vial; once flies began to eclose from a vial, the number of adults was counted every 4 h. Development time was estimated as the number of hours from oviposition to adult eclosion, and egg-to-adult viability was assessed as the proportion of eggs laid that eclosed as adults. Data were analyzed with ANOVA, with estimates of egg-to-adult viability being arcsine-square-root transformed prior to analysis.

Diapause Phenotyping: Field Collections

Isofemale lines were sampled from rural and urban environments to evaluate temporal changes in the frequency of diapause phenotypes. The rural collections were done at Solebury Orchards (New Hope, PA) and Terhune Orchards (Princeton, NJ) in May, July, and September in both 2003 and 2004. Flies were collected by a combination of baiting and sweep netting and sorted into isofemale lines in the laboratory. A minimum of 70 lines per population per sampling time were used for phenotypic characterizations. Species identification was established by examining resulting male progeny. Approximately 10 females were collected from each line, placed under diapause-inducing conditions, and phenotyped 4 weeks later. The urban collections were made from Yards Brewing Company (Philadelphia, PA) and an indoor/outdoor produce market in South Philadelphia. Both locations contain large breeding *D. melanogaster* populations throughout the year. Urban flies were collected within 2 days of the rural collections at all time points and phenotyped simultaneously. Data for diapause incidence were analyzed with nominal logistic regression as before.

RESULTS

Changes in Diapause Incidence

The experimental manipulation had a significant effect on the proportion of flies from a cage that expressed reproductive diapause (Fig. 1). In the F_1 generation, the mean frequency of diapause was 0.76 (range 0.65–0.86). Assuming random mating among virgin flies used to establish the cages, this is consistent with a predicted 3:1 ratio if diapause segregates as an autosomal dominant (Schmidt et al. 2005a). In the control cages, the frequency of nondiapause genotypes had significantly increased by the fourth generation and continued to increase thereafter at a slower rate. In contrast, the frequency of diapause genotypes increased subsequent to exposure to both starvation and cold stress. These patterns were consistent across replicate cages and between the two independent genetic backgrounds (Table 1). At the end of experiment, following the last exposure to environmental stress, the odds of a fly in a stress cage expressing diapause relative to nondiapause were 65.24 times higher than in the control

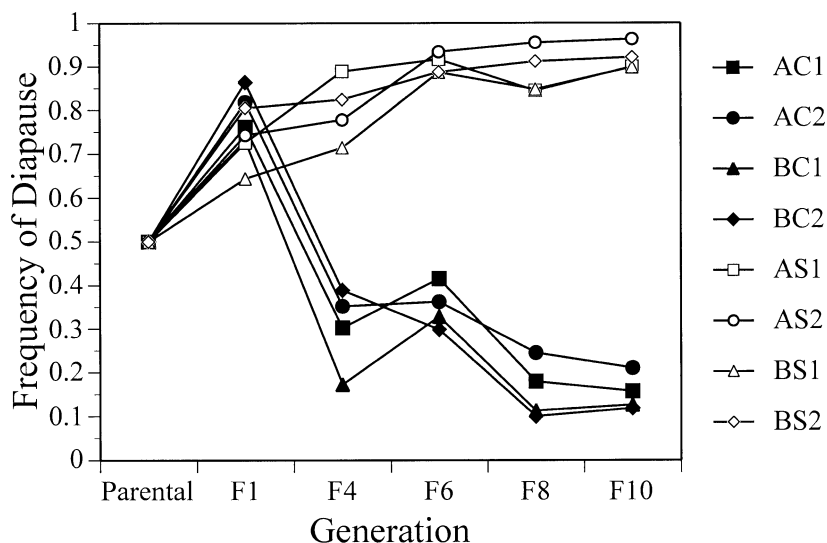


FIG. 1. Change in the frequency of diapause incidence over generational time in the eight experimental population cages. A and B denote genetic background; C and S denote the control and stress treatments, respectively. Diapause frequency was assayed in the first filial generation and in each generation following exposure to starvation or cold stress. Starvation stress was applied in the third and seventh generations, and cold stress in the fifth and ninth generations.

cages. The lower and upper 99% confidence intervals around this odds ratio were 37.45 and 119.20, respectively.

Diapause incidence was also observed to change over time in the collections of isofemale lines from rural orchard populations (Fig. 2). Analysis indicated significant effects of date of collection (Wald $\chi^2 = 83.18$, $df = 5$, $P < 0.001$), habitat (Wald $\chi^2 = 358.24$, $df = 1$, $P < 0.001$), and the interaction term (Wald $\chi^2 = 33.21$, $df = 5$, $P < 0.001$). The frequency of diapause incidence was higher in the first collection following the winter season than in late summer, and the observed seasonal variation was greatly magnified in the orchard relative to urban populations (Table 2). These patterns were consistent in both sampled years.

Life Histories

Initial estimates for longevity indicated no widespread differences among cages or treatments, but a significant interaction between genetic background and sex was observed (Table 3). Mean life span was similar between genetic backgrounds for the female sex (A females: 49.4 days; B females: 47.7 days), whereas mean life span for males differed between the A and B genetic backgrounds (40.5 and 53.8 days, respectively). In contrast to these differences observed in initial life span, the experimental manipulation significantly impacted female life span only (Table 3). Life expectancies for males were equivalent between the stress and control

treatments at the end of the experiment, but females from the stress cages exhibited a 27.8% life span extension relative to females from control cages (means plotted in Fig. 4).

Similar to the data on longevity, there was no significant variation in total or per capita fecundity among cages when assayed in the second generation, prior to experimental manipulation (not shown). At the end of the experimental period, as assayed in the 13th generation of culture, there was a slight but nonsignificant difference in total lifetime fecundity between treatments: mean fecundity for control females was 1370 eggs, and the mean for females in the stress treatment was 1241 ($F_{1,136} = 1.435$, $P > 0.233$). However, per capita fecundity was higher for control females for the first 36 days of life, and differences between treatments were highest early in life (Fig. 3). The difference between treatments was non-randomly distributed over time by a runs test ($n_1 = 66$, $n_2 = 9$, eight runs, $P < 0.025$; Sokal and Rohlf 1981).

The experimental manipulation had more widespread effects on standardized lipid content than on dry weight (Table 4). Total body lipid content for males was statistically indistinguishable between treatments (within model planned comparison: $F_{1,179} = 0.013$, $P < 0.909$), but females from the stress treatment had a higher proportion of body weight comprised of lipid than did control females ($F_{1,179} = 10.49$, $P < 0.001$). Lipid content was positively correlated with female but not male life span (Fig. 4A). Mean dry weight

TABLE 1. Maximum likelihood parameter estimates for the logistic regression analysis modeling the log odds (dispause/nondiapause) in the 10th generation in the experimental demography cages.

Term	Estimate	SE	χ^2	P	Odds ratio
Intercept	0.355	0.112	10.01	0.0016	
Treatment	2.089	0.112	347.64	0.00001	65.24*
Genetic background	0.185	0.112	2.73	0.098	1.45
Genetic background \times treatment	-0.0543	0.112	0.23	0.628	0.897

* The 99% confidence intervals do not include 1.0.

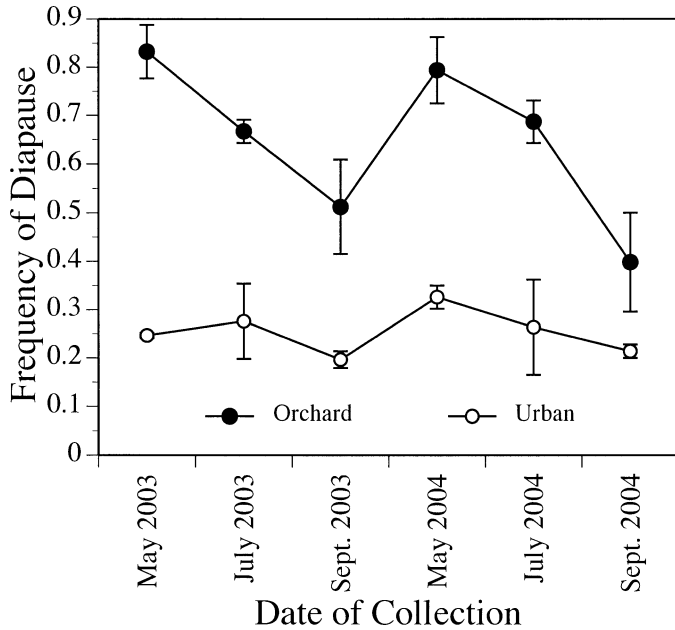


FIG. 2. Change in the frequency of diapause incidence (mean \pm SE) over time in Pennsylvania orchard (O) and urban (U) isofemale line populations.

was equivalent between treatments but differed between sexes, as would be expected. Although neither genetic background nor treatment had significant effects on weight, flies of the A genetic background were heavier in the control treatment, whereas flies of the B genetic background were heavier in the stress treatment. These differences are reflected in the significant genetic background-by-treatment interaction term.

Development time as well as transformed egg-to-adult viability also diverged between treatments (Table 5; Fig. 4B). Mean time to eclosion was 18.4 h longer for flies from the stress relative to the control treatment. However, the mean proportion of eggs laid that successfully eclosed as adults was 19.6% higher in the stress compared to control treatment. Genetic background also had a significant effect on egg-to-adult viability, as flies of the B background exhibited slightly higher (8.3%) mean viability.

DISCUSSION

Drosophila melanogaster is a tropical insect that has colonized temperate habitats in North America in the relatively recent past (David and Capy 1988). Temperate populations persist over time by means of adult overwintering, which has been associated with delays in the onset of reproduction in both European (e.g., Boulétreau-Merle and Fouillet 2002) and Australian (Mitrovski and Hoffmann 2001) populations. Diapause expression is a widespread mechanism for overwintering in insect taxa that occupy seasonal environments, including temperate species of *Drosophila* (e.g., Lumme and Lakovaara 1983; Kimura 1988b). The life-span extension, reduction in age-specific mortality rates, and greatly increased stress resistance that accompany diapause expression indicate that this trait is also functionally associated with overwintering in the cosmopolitan species *D. melanogaster* (Tatar et al. 2001).

This inference predicts that diapause frequency should be positively associated with the degree of seasonality and severity of stress experienced in a given habitat during unfavorable periods. This was observed, as diapause incidence is clinal across the latitudinal gradient in the eastern United States (Schmidt et al. 2005a). Given the potential fitness advantages of diapause expression during prolonged periods of environmental stress (e.g., Lumme and Lakovaara 1983; Kimura 1988a; Tatar et al. 2001), it is not surprising that diapause incidence is at high frequency in northern temperate populations. Substantial gene flow among populations in the eastern United States is expected based on both direct estimates of dispersal (e.g., Coyne and Milstead 1987) and indirect estimates from the geographic distribution of presumably neutral genetic markers (e.g., Hale and Singh 1991; Berry and Kreitman 1993; Sezgin et al. 2004). This may, in part, explain the occurrence and persistence of nondiapause genotypes in temperate regions. What remains unclear is why diapause-expressing genotypes are at relatively low frequency in southern neotropical locales. In such habitats, *Drosophila* populations are not exposed to temperatures that would both induce and maintain reproductive quiescence for long periods of time; diapause expression may not be required for overwinter survivorship of individuals or associated with population persistence.

TABLE 2. Maximum likelihood parameter estimates for the logistic regression modeling the log odds (diapause/nondiapause) in seasonal collections of isofemale lines from Pennsylvania orchard and urban habitats.

Term	Estimate	SE	χ^2	P	Odds ratio
Intercept	-0.2084	0.0467	19.88	0.0001	
Date (July 2003)	0.0665	0.101	0.44	0.508	1.14
Date (July 2004)	0.0868	0.102	0.73	0.393	1.19
Date (May 2003)	0.438	0.112	15.26	0.0001	2.41*
Date (May 2003)	0.521	0.105	24.43	0.0001	2.83*
Date (Sept. 2003)	-0.471	0.104	20.44	0.0001	0.39*
Habitat (orchard)	0.885	0.0467	358.24	0.0001	5.86*
Date (July 2003) \times habitat (orchard)	-0.0571	0.101	0.32	0.569	0.89
Date (July 2004) \times habitat (orchard)	0.0138	0.102	0.02	0.892	1.03
Date (May 2003) \times habitat (orchard)	0.471	0.112	17.59	0.0001	2.56*
Date (May 2004) \times habitat (orchard)	0.158	0.105	2.26	0.133	1.37
Date (Sept. 2003) \times habitat (orchard)	-0.146	0.104	1.96	0.161	0.75

* The 99% confidence intervals do not include 1.0.

TABLE 3. Proportional hazards analyses of life span.

Source	df	Initial		Final	
		LR χ^2	P	LR χ^2	P
Genetic background	1	1.234	0.267	0.0036	0.953
Treatment	1	0.213	0.644	5.771	0.016
Sex	1	0.198	0.656	1.967	0.161
Genetic background \times treatment	1	0.352	0.553	0.032	0.858
Genetic background \times sex	1	5.797	0.016	0.234	0.629
Treatment \times sex	1	1.945	0.163	5.883	0.015

Schmidt et al. (2005a) hypothesized that the observed cline in diapause incidence reflects fitness trade-offs associated with the observed variance for diapause expression. Subsequent characterizations of inbred lines derived from a single temperate source population documented the potential for fitness trade-offs. First, diapause genotypes were constitutively longer lived and had reduced rates of age-specific mortality, higher preadult viability, and enhanced resistance to both cold and starvation stress. Second, nondiapause genotypes were significantly more fecund early in life and developed from egg to adult at a faster rate. Finally, for the life-history traits investigated, the resulting genetic variance-covariance matrices were significantly distinct between diapause and nondiapause inbred lines (Schmidt et al. 2005b). The observed life-history variation between diapause and nondiapause lines is similar in many respects to the variation between starvation-selected and control lines (e.g., Harshman et al. 1999).

Experimental Population Cages

The major goal of the experiments presented here was to test the hypothesis that the relative fitness of genotypes that express and do not express reproductive diapause in the standard laboratory assay varies with environment in a manner predicted by their life-history profiles and genetic variance-covariance matrices. It was predicted that diapause genotypes

would be of higher relative fitness in environments characterized by some degree of environmental stress, as these genotypes are more stress resistant even in the absence of diapause expression. Conversely, nondiapause genotypes were predicted to be of higher fitness in more benign environments, as standard culture under optimal conditions for a holometabolous insect is predicted to result in strong selection on development rate and early life fecundity (e.g., Chippindale et al. 1997; Sgró and Partridge 2000) as well as the timing of oviposition (Gilpin 1974).

The results presented here are consistent with these predictions. In the laboratory, populations cultured under the two distinct environmental regimes significantly diverged for a series of life-history traits over a relatively short temporal interval. After 13 generations and exposure to four bouts of moderate environmental stress, populations in the stress treatment were characterized by a higher frequency of reproductive diapause incidence, female life-span extension, and higher lipid content. The sex-specific effects of selection observed in this experiment are consistent with the results observed by Chippindale et al. (1996).

It remains unclear what actual traits were selected to generate the results observed here. Exposure to starvation stress has been hypothesized to select on a variety of traits including metabolic rate and/or lipid content (Hoffmann and Parsons 1991). In selecting directly on starvation resistance, Harshman et al. (1999) observed no change in resting metabolic rate between selected and control lines, whereas the selected lines demonstrated an increase in body weight, triacylglyceride content, and enzyme activities associated with lipid biogenesis. Selection on starvation resistance also resulted in a longer development time from egg to adult; similar results were observed by Chippindale et al. (1996), and this phenotypic correlation is also evident in the results presented here. Given the established links between lipid content and starvation resistance (e.g., Service 1987; Chippindale et al. 1996; Harshman et al. 1999; Hoffmann et al. 2005b) and the increased lipid content of females in the stress compared to the control treatment, exposure to starvation stress may have selected directly on lipid content. However, although diapause inbred lines were previously observed to be more resistant to starvation stress, these lines were characterized by a slight but significant reduction in total body lipid content compared to the nondiapause inbred lines analyzed (Schmidt et al. 2005b). It remains unknown how total body lipid content is partitioned into distinct lipid source pools for these sets of diapause and nondiapause inbred lines. Preliminary results have demonstrated a consistently lower basal meta-

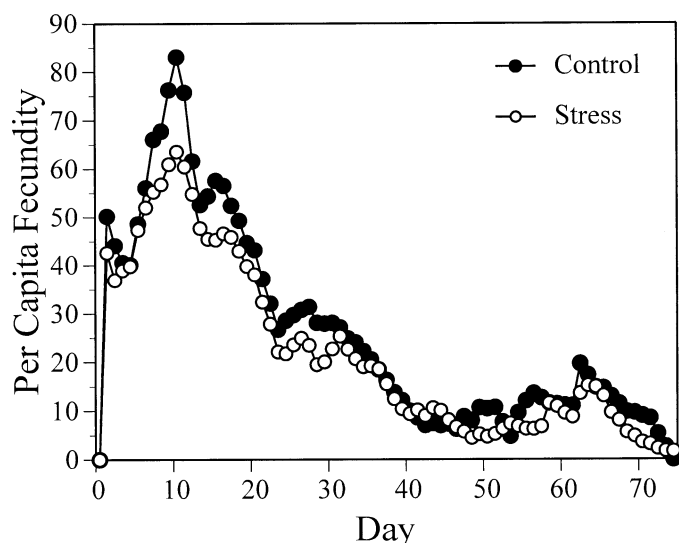


FIG. 3. Per capita fecundity for females in the control and stress cages. Data are based on a sliding window of 3-day means.

TABLE 4. Analysis of variance for total body lipid content and body weight in experimental cages.

Source	df	Lipid content		Dry weight	
		SS × 10 ⁹	F	SS × 10 ⁹	F
Genetic background	1	0.028	0.0001	21.99	0.268
Treatment	1	6.874	668.955*	8.008	0.0995
Sex	1	0.961	1.464	685.631	188.516*
Genetic background × treatment	1	0.0104	0.0083	80.451	39.699***
Genetic background × sex	1	0.649	0.521	3.658	1.805
Treatment × sex	1	6.841	5.498*	0.0945	0.4665
Error	178	221.53		360.687	

* $P < 0.05$; *** $P < 0.0001$.

bolic rate across adult age classes and temperatures for diapause relative to nondiapause genotypes (C. Wills and P. Schmidt, unpubl. data).

In comparison to selection on starvation resistance, the basis for selection on cold resistance is not as well understood. Resistance to cold shock is increased by short-term chilling or cold hardening (e.g., Chen and Walker 1993; Kelty and Lee 1999; Ayrinhac et al 2004; Anderson et al. 2005), but the variation in cold resistance among populations is not solely due to plasticity (e.g., Bublly et al 2002). In natural populations sampled across latitudinal gradients, temperate populations are consistently more resistant to various forms of cold stress than are tropical populations (Guerra et al. 1997; Gibert et al. 2001; Bublly et al. 2002; Hoffmann et al. 2002a; Ayrinhac et al. 2004; Hoffmann et al. 2005a). Although a positive phenotypic correlation between lipid content and cold resistance was observed by Hoffmann et al. (2001), evidence exists for a trade-off between cold resistance and starvation resistance (Hoffmann et al. 2005b) as well as between low and high temperature resistance (Hoffmann et al. 2002). Hoffmann et al. (2005b) selected directly on either starvation or cold resistance; lines selected for starvation resistance were characterized by higher lipid content and increased desiccation resistance but were less cold resistant. Similarly, lines selected for cold resistance demonstrated a decrease in resistance to starvation stress and did not exhibit the increase in lipid content that is commonly observed in starvation-selected lines (e.g., Chippindale et al. 1996; Harshman et al. 1999).

In the present experiment, experimental flies were not rapidly cold hardened prior to stress exposure and resistance was based on cold shock, not chill coma recovery (e.g., Macdonald et al. 2004). Lines selected for decreased chill coma recovery time also demonstrate increased resistance to severe cold shock (Anderson et al. 2005), and selection on cold shock resistance is more effective in the absence of prior cold hardening (Watson and Hoffmann 1996). While rapid cold hardening may be associated with changes in lipid content or composition (e.g., Overgaard et al. 2005), selection based on resistance to extreme cold shock may be more related to constitutive variation in polyol content (e.g., Yoder et al. 2006) or carbohydrate cryoprotectants (e.g., Kelty and Lee 2001). Thus, assuming that the observed trade-off between starvation and cold resistance is mediated by lipid metabolism (Hoffman et al. 2005b), exposure of flies to both starvation and cold stress in the present experiment would be predicted to result in distinct if not counter selection pressures

on the underlying life-history trait variation. Although selection on starvation and cold resistance may have differential effects on lipid content and correlated traits, exposure to both stresses caused a similar increase in the frequency of diapause incidence observed here.

Seasonal Changes in Diapause Incidence

The robust life-history differentiation between diapause genotypes allows a further prediction regarding temporal variation in frequency. In seasonal environments the frequency of genotypes that express diapause should be high following the winter, assuming that diapause expression is positively associated with survivorship over this period. If these genotypes are of lower relative fitness under conditions conducive to *Drosophila* reproduction and population growth, the frequency of diapause incidence should decline over the summer months. In contrast, the frequency of genotypes that express diapause should be relatively low and constant in environments that exhibit a lower degree of seasonality with regard to temperature and food availability. The results presented here are also consistent with these predictions. In resident orchard populations that experience pronounced seasonality, the frequency of diapause incidence was observed to cycle over time. In urban environments in which flies maintain breeding populations throughout the year, diapause frequency was low and seasonally homogeneous. No effort was made to quantify the degree of seasonality in the urban habitats sampled or the magnitude of the difference in relevant environmental parameters that *Drosophila* populations experience in the orchard versus urban habitats. Habitat type was a qualitative designation based on the observed seasonal variation in reproductive activity. In the orchards sampled, adult *D. melanogaster* can be collected by baiting and trapping from March through December only; in contrast, in a variety of human-associated urban habitats, including the ones sampled in the present study, gravid *D. melanogaster* females are abundant throughout the year (P. Schmidt, pers. obs.).

Relation to the Cline

A simple hypothesis for geographic variation in diapause incidence in *D. melanogaster* can be derived from the theoretical treatment of the timing of diapause by Cohen (1970) and its modification by Kimura (1988a). The onset of diapause expression, and by extension the frequency of diapause genotypes, in a given habitat is predicted to be determined

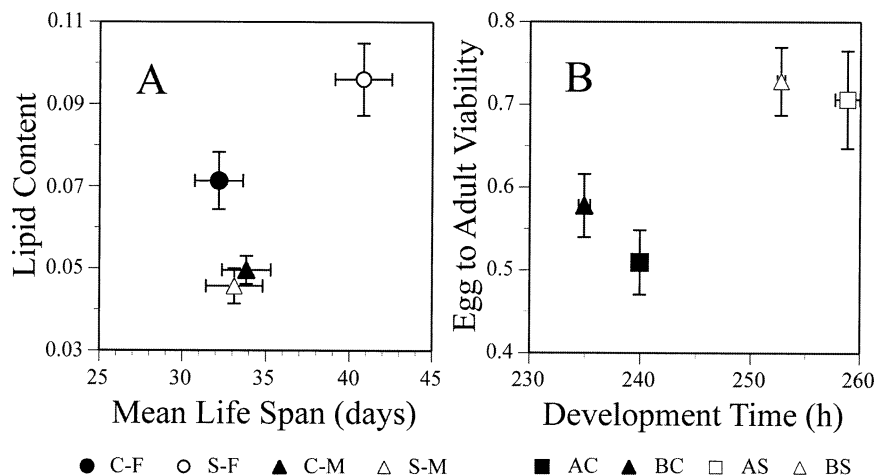


FIG. 4. (A) Phenotypic association between lipid content and life span for males and females in the control and stress treatments. (B) Phenotypic association between preadult viability and development time for genetic background by treatment combinations. All datapoints are plotted as a mean \pm standard error.

by three factors that occur over the winter season: (1) the probability of successful reproduction; (2) the probability of survival without diapause expression; and (3) the probability of survival while in diapause. Diapause genotypes should be at high frequency when the gain in fitness associated with diapause expression (relative overwintering survivorship of diapause to nondiapause genotypes) is greater than the associated cost (reproductive quiescence). Given that *D. melanogaster* larvae cannot tolerate continual exposure to low temperatures (e.g., Kimura 1988a), the probability of successful reproduction during the temperate winter is presumed to be negligible. Furthermore, early-winter reproduction was shown to be entirely unsuccessful in Australian populations reared in outdoor population cages (Mitrovski and Hoffmann 2001). The elevated frequency of diapause genotypes following the winter season in temperate orchards observed here suggests that nondiapause genotypes have low overwintering survivorship relative to diapause genotypes in temperate habitats.

The current study did not directly address why the frequency of reproductive diapause incidence varies substantially with latitude in eastern U.S. populations. However, these experiments address the maintenance of the cline indirectly by evaluating fitness trade-offs between diapause and nondiapause genotypes in the laboratory environment. In northern temperate populations, such as the Walpole, Maine population from which these inbred lines were derived (Schmidt et al. 2005b), overwintering survivorship must involve substantial life-span extension and somatic maintenance

under stressful conditions of below-freezing temperatures and reduced food availability. Here, exposure to moderate stress was sufficient to greatly increase the frequency of genotypes that express reproductive diapause. This occurred in the absence of cues that would elicit and maintain diapause expression. The actual expression of reproductive diapause in *D. melanogaster* causes significant increases in resistance to multiple forms of environmental stress (Tatar et al. 2001), as it does in other species of *Drosophila* (e.g., Kimura 1988a). Thus, the results suggest that diapause genotypes are of higher relative fitness in stressful environments and that the fitness differences between diapause and nondiapause genotypes may be exacerbated when diapause is induced and maintained by the shortened photoperiod and low temperatures that characterize the temperate winter season.

The life-history trade-off hypothesis for the maintenance of the diapause cline predicts that nondiapause genotypes are of higher relative fitness in less stressful environments. The results of the population cage experiment are informative in this respect, as they suggest specific costs associated with diapause genotypes. Nondiapause genotypes are more fecund early in life and have a reduced development time (Schmidt et al. 2005b); differences in patterns and timing of development between diapause and nondiapause strains have also been reported for other taxa (Hoy and Knopf 1978; Wipking and Kurtz 2000). In the present experiment, culture under standard laboratory conditions resulted in a rapid and significant decrease in the frequency of diapause genotypes.

TABLE 5. Analysis of variance for development time and transformed percent viability.

Source	Development time			Egg to adult viability		
	df	SS	F	df	SS	F
Genetic background	1	15.335	64.822	1	0.221	173.003*
Treatment	1	174.314	736.859*	1	1.053	822.993*
Genetic background \times treatment	1	0.237	0.948	1	0.0013	0.014
Error	2344	585.131		127	11.287	

* $P < 0.05$.

Associated with this change, individuals from the control populations were more fecund and developed from egg to adult at a significantly faster rate. Culture under laboratory conditions may also be associated with a reduction or loss of the diapause response in other insects (e.g., Glass 1970). Similarly, fitness trade-offs associated with diapause expression and the timing of diapause entry are inferred in the *D. auraria* species complex in Japan (Kimura 1988a).

In summary, our results indicate that the relative fitness of diapause genotypes in *D. melanogaster* varies substantially and predictably with environment. This suggests the latitudinal cline in diapause incidence among populations and the seasonal variation within temperate habitats reflects this context dependency to relative fitness, but this was not directly addressed in the present study. The manipulative experiment was conducted solely in the laboratory environment, and it is unknown how the observed patterns may reflect population dynamics in a natural setting. Currently, field-based experiments are being conducted to address this question. In conjunction with controlled manipulations in the laboratory, such direct fitness estimates will help elucidate whether the environmental heterogeneity experienced by *Drosophila* populations in the wild may actively maintain the genetic variation underlying the observed variance in reproductive diapause expression.

ACKNOWLEDGMENTS

We thank A. Paaby for assistance in the field and isofemale line establishment. We also extend thanks to three anonymous reviewers for constructive comments on a previous version of the manuscript. This work was supported by grant DEB 0236577 from the U.S. National Science Foundation.

LITERATURE CITED

- Anderson, A. R., A. A. Hoffmann, and S. W. McKechnie. 2005. Response to selection for rapid chill-coma recovery in *Drosophila melanogaster*: physiology and life-history traits. *Genet. Res.* 85(1):15–22.
- Ayrinhac, A., V. Debat, P. Gibert, A. G. Kister, H. Legout, B. Moreteau, R. Vergilino, and J. R. David. 2004. Cold adaptation in geographical populations of *Drosophila melanogaster*: phenotypic plasticity is more important than genetic variability. *Funct. Ecol.* 18(5):700–706.
- Berry, A., and M. Kreitman. 1993. Molecular analysis of an allozyme cline: alcohol dehydrogenase in *Drosophila melanogaster* on the east coast of North America. *Genetics* 134:869–893.
- Boulétreau-Merle, J., and P. Fouillet. 2002. How to overwinter and be a founder: egg retention phenotypes and mating status in *Drosophila melanogaster*. *Evol. Ecol.* 16:309–322.
- Boulétreau-Merle, J., R. Allemand, Y. Cohet, and J. R. David. 1982. Reproductive strategy in *Drosophila melanogaster*: significance of a genetic divergence between temperate and tropical populations. *Oecologia* 53:323–329.
- Bubli, O. A., A. Riihimaa, F. M. Norry, and V. Loeschcke. 2002. Variation in resistance and acclimation to low-temperature stress among three geographical strains of *Drosophila melanogaster*. *J. Therm. Biol.* 27(5):337–344.
- Chen, C. P., and V. K. Walker. 1993. Increase in cold-shock tolerance by selection of cold resistant lines in *Drosophila melanogaster*. *Ecol. Entomol.* 18(3):184–190.
- Chippindale, A. K., T. J. F. Chu, and M. R. Rose. 1996. Complex trade-offs and the evolution of starvation resistance in *Drosophila melanogaster*. *Evolution* 50:753–766.
- Chippindale, A. K., J. A. Alipaz, H.-W. Chen, and M. R. Rose. 1997. Experimental evolution of accelerated development in *Drosophila*. 1. Developmental speed and larval survival. *Evolution* 51(5):1536–1551.
- Cohen, D. 1970. A theoretical model for the optimal timing of diapause. *Am. Nat.* 104:389–400.
- Coyne, J. A., and B. Milstead. 1987. Long distance migration in *Drosophila*. 3. Dispersal of *D. melanogaster* alleles from a Maryland orchard. *Am. Nat.* 130:70–82.
- Danilevskii, A. S. 1965. Photoperiodism and seasonal development of insects. Oliver and Boyd, London.
- David, J. R., and P. Capi. 1988. Genetic variation of *Drosophila melanogaster* natural populations. *Trends in Genet.* 4:106–111.
- Endler, J. A. 1986. Natural selection in the wild. Princeton Univ. Press, Princeton, NJ.
- Gibert, P., B. Moreteau, G. Petavy, D. Karan, and J. R. David. 2001. Chill-coma tolerance, a major climatic adaptation among *Drosophila* species. *Evolution* 55(5):1063–1068.
- Gilpin, M. E. 1974. Intraspecific competition between *Drosophila* larvae in serial transfer systems. *Ecology* 55(5):1154–1159.
- Glass, E. H. 1970. Changes in diapause response to photoperiod in laboratory strains of Oriental fruit moth. *Ann. Entomol. Soc. Amer.* 63:74–76.
- Gockel, J., W. J. Kennington, A. Hoffmann, D. B. Goldstein, and L. Partridge. 2001. Nonclinality of molecular variation implicates selection in maintaining a morphological cline of *Drosophila melanogaster*. *Genetics* 158:319–323.
- Guerra, D., S. Cavicchi, R. A. Krebs, and V. Loeschcke. 1997. Resistance to heat and cold stress in *Drosophila melanogaster*: intra- and inter-population variation in response to climate. *Genet. Sel. Evol.* 29(5):497–510.
- Hale, L. R., and R. S. Singh. 1991. Contrasting patterns of genetic structure and evolutionary history as revealed by mitochondrial DNA and nuclear gene-enzyme variation. *J. Genet.* 70:79–89.
- Harshman, L. G., A. A. Hoffman, and A. G. Clark. 1999. Selection for starvation resistance in *Drosophila melanogaster*: physiological correlates, enzyme activities and multiple stress responses. *J. Evol. Biol.* 12:370–379.
- Hoffmann, A. A., and P. A. Parsons. 1991. Evolutionary genetics and environmental stress. Oxford Univ. Press, Oxford, U.K.
- Hoffmann, A. A., R. Hallas, C. Sinclair, and P. Mitrovski. 2001. Levels of variation in stress resistance in *Drosophila* among strains, local populations, and geographic regions: patterns for desiccation, starvation, cold resistance, and associated traits. *Evolution* 55(8):1621–1630.
- Hoffmann, A. A., A. Anderson, and R. Hallas. 2002. Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecol. Lett.* 5:614–618.
- Hoffmann, A. A., M. Scott, L. Partridge, and R. Hallas. 2003. Overwintering in *Drosophila melanogaster*: outdoor field cage experiments on clinal and laboratory selected populations help elucidate traits under selection. *J. Evol. Biol.* 16:614–623.
- Hoffmann, A. A., J. Shirriffs, and M. Scott. 2005a. Relative importance of plastic vs. genetic factors in adaptive differentiation: geographical variation for stress resistance in *Drosophila melanogaster* from eastern Australia. *Funct. Ecol.* 19(2):222–227.
- Hoffmann, A. A., R. Hallas, A. R. Anderson, and M. Telonis-Scott. 2005b. Evidence for a robust sex-specific trade-off between cold resistance and starvation resistance in *Drosophila melanogaster*. *J. Evol. Biol.* 18(4):804–810.
- Hoy, M. A., and N. F. Knop. 1978. Development hatch dates, overwintering success, and spring emergence of a non-diapausing gypsy moth strain (Lepidoptera Orgyidae) in field cages. *Canad. Entomol.* 110(9):1003–1008.
- Karan, D., N. Dahiya, A. K. Munjal, P. Gibert, B. Moreteau, R. Parkash, and J. R. David. 1998. Desiccation and starvation tolerance of adult *Drosophila*: opposite latitudinal clines in natural populations of three different species. *Evolution* 52:825–831.
- Kelty, J. D., and R. E. Lee. 1999. Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. *J. Insect Physiol.* 45(8):719–726.
- . 2001. Rapid cold hardening of *Drosophila melanogaster* (Diptera: Drosophilidae) during ecologically based thermoperiodic cycles. *J. Exp. Biol.* 204(9):1659–1666.

- Kimura, M. T. 1988a. Adaptations to temperate climates and evolution of overwintering strategies in the *Drosophila melanogaster* species group. *Evolution* 42(6):1288–1297.
- . 1988b. Interspecific and geographic variation of diapause intensity and seasonal adaptation in the *Drosophila auraria* species complex (Diptera: Drosophilidae). *Funct. Ecol.* 2:177–183.
- King, R. C. 1970. Ovarian development in *Drosophila melanogaster*. Academic Press, New York.
- Lumme, J., and S. Lakovaara. 1983. Seasonality and diapause in Drosophilids. Pp. 171–220 in *Genetics and biology of Drosophila*, M. Ashburner, H. L. Carson, and J. N. J. Thompson, eds. Academic Press, London.
- Macdonald, S. S., L. Rako, P. Batterham, and A. A. Hoffmann. 2004. Dissecting chill coma recovery as a measure of cold resistance: evidence for a biphasic response in *Drosophila melanogaster*. *J. Insect Physiol.* 50(8):695–700.
- Mitrovski, P., and A. A. Hoffmann. 2001. Postponed reproduction as an adaptation to winter conditions in *Drosophila melanogaster*: evidence for clinal variation under semi-natural conditions. *Proc. R. Soc. Lond. B* 268:2163–2168.
- Oakeshott, J. B., P. R. Anderson, W. R. Knibb, D. G. Anderson, and G. K. Chambers. 1982. Alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase clines in *Drosophila melanogaster* on different continents. *Evolution* 36:86–96.
- Overgaard, J., J. G. Sorensen, S. O. Petersen, V. Loeschcke, and M. Holmstrup. 2005. Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *J. Insect Physiol.* 51(11):1173–1182.
- Richard, D. S., N. L. Watkins, R. B. Serafin, and L. I. Gilbert. 1998. Ecdysteroids regulate yolk protein uptake by *Drosophila melanogaster* oocytes. *J. Insect Physiol.* 44:637–644.
- Richard, D. S., J. M. Jones, M. R. Barbarito, S. Cerula, J. P. De-tweiler, S. J. Fisher, D. M. Brannigan, and D. M. Scheswohl. 2001. Vitellogenesis in diapausing and mutant *Drosophila melanogaster*: further evidence for the relative roles of ecdysteroids and juvenile hormones. *J. Insect Physiol.* 47:905–913.
- Robinson, S. J. W., B. Zwaan, and L. Partridge. 2000. Starvation resistance and adult body composition in a latitudinal cline of *Drosophila melanogaster*. *Evolution* 54:1819–1824.
- Saunders, D. S. 1990. The circadian basis of ovarian diapause regulation in *Drosophila melanogaster*: Is the period gene causally involved in photoperiodic time measurement? *J. Biol. Rhythms* 5:315–331.
- Saunders, D. S., and L. I. Gilbert. 1990. Regulation of ovarian diapause in *Drosophila melanogaster* by photoperiod and moderately low temperature. *J. Insect Physiol.* 36:195–200.
- Saunders, D. S., V. C. Henrich, and L. I. Gilbert. 1989. Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proc. Nat. Acad. Sci. USA* 86:3748–3752.
- Saunders, D. S., D. S. Richard, S. W. Applegaum, and L. I. Gilbert. 1990. Photoperiodic diapause in *Drosophila melanogaster* involves a block to the juvenile hormone regulation of ovarian maturation. *Gen. Comp. Endocrin.* 79:174–184.
- Schmidt, P. S., D. D. Duvernell, and W. F. Eanes. 2000. Adaptive evolution of a candidate gene for aging in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 97:10861–10865.
- Schmidt, P. S., L. M. Matzkin, M. Ippolito, and W. F. Eanes. 2005a. Geographic variation in diapause incidence, life-history traits, and climatic adaptation in *Drosophila melanogaster*. *Evolution* 59(8):1721–1732.
- Schmidt, P. S., A. B. Paaby, and M. S. Heschel. 2005b. Genetic variance for diapause expression and associated life histories in *Drosophila melanogaster*. *Evolution* 59(12):2616–2625.
- Service, P. M. 1987. Physiological mechanisms of increased stress resistance in *Drosophila melanogaster* selected for postponed senescence. *Physiol. Zool.* 60:321–326.
- Sezgin, E., D. D. Duvernell, L. M. Matzkin, Y. Duan, C.-T. Zhu, B. C. Verrelli, and W. F. Eanes. 2004. Single locus latitudinal clines in metabolic genes, derived alleles, and their relationship to temperate adaptation in *Drosophila melanogaster*. *Genetics* 168(2):923–931.
- Sgrò, C. M., and L. Partridge. 2000. Evolutionary responses of the life history of wild-caught *Drosophila melanogaster* to two standard methods of laboratory culture. *Am. Nat.* 156(4):341–353.
- Tatar, M., S. Chien, and N. K. Priest. 2001. Negligible senescence during reproductive dormancy in *Drosophila melanogaster*. *Am. Nat.* 158(3):248–258.
- Tauber, M. J., C. A. Tauber, and S. Masaki. 1986. Seasonal adaptations of insects. Oxford Univ. Press, New York.
- Watson, M. J. O., and A. A. Hoffmann. 1996. Acclimation, cross-generation effects, and the response to selection for increased cold resistance in *Drosophila*. *Evolution* 50(3):1182–1192.
- Williams, K. D., and M. B. Sokolowski. 1993. Diapause in *Drosophila melanogaster* females: a genetic analysis. *Heredity* 71:312–317.
- Wipking, W., and J. Kurtz. 2000. Genetic variability in the diapause response of the burnet moth *Zygaena trifolii* (Lepidoptera: Zygaenidae). *J. Insect Physiol.* 46(2):127–134.
- Yoder, J. A., J. B. Benoit, D. L. Denlinger, and D. B. Rivers. 2006. Stress-induced accumulation of glycerol in the flesh fly, *Sarcophaga bullata*: evidence indicating anti-desiccant and cryoprotectant functions of this polyol and a role for the brain in coordinating the response. *J. Insect Physiol.* 52(2):202–214.

Corresponding Editor: H. Hollocher