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GEOGRAPHIC VARIATION IN DIAPAUSE INCIDENCE, LIFE-HISTORY TRAITS, AND CLIMATIC ADAPTATION IN *DROSOPHILA MELANOGASTER*

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Abstract.—In *Drosophila melanogaster*, exposure of females to low temperature and shortened photoperiod can induce the expression of reproductive quiescence or diapause. Diapause expression is highly variable within and among natural populations and has significant effects on life-history profiles, including patterns of longevity, fecundity, and stress resistance. We hypothesized that if diapause expression is associated with overwintering mechanisms and adaptation to temperate environments, the frequency of diapause incidence would exhibit a latitudinal cline among natural populations. Because stress resistance and reproductive traits are also clinal in this species, we also examined how patterns of fecundity and longevity varied with geography and how stress resistance and associated traits differed constitutively between diapause and nondiapause lines. Diapause incidence was shown to vary predictably with latitude, ranging from 35% to 90% among natural populations in the eastern United States Survivorship under starvation stress differed between diapause and nondiapause lines; diapause phenotypes were also distinct for total body triglyceride content and the developmental distribution of oocytes in the ovary following stress exposure. Patterns of longevity, fecundity, and ovariole number also varied with geography. The data suggest that, for North American populations, diapause expression is functionally associated with overwintering mechanisms and may be an integral life-history component in natural populations.

Key words.—Cline, diapause, *Drosophila*, life history.

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The role of genetic variation in the process of adaptation to environmental heterogeneity is a fundamental issue in evolutionary biology. Across broad geographic scales, latitudinal clines in the frequency of specific alleles and/or phenotypes have long been used to infer adaptation to climatic variation in an array of taxa. Among *Drosophila melanogaster* populations along latitudinal transects, alleles at several loci demonstrate significant clines in frequency (Oakeshott et al. 1982; Berry and Kreitman 1993; Duvernell and Eanes 2000; Schmidt et al. 2000; Verrelli and Eanes 2001; Bettencourt et al. 2002; Frydenberg et al. 2003; Sezgin et al. 2004). Similar associations have been observed for chromosomal inversions (e.g., Knibb 1982). By themselves, such patterns may simply reflect genetic structure along the environmental gradient. However, polymorphisms thought to be neutral with respect to fitness, such as synonymous site and mitochondrial DNA (mtDNA) variants, are not clinal (Hale and Singh 1991; Berry and Kreitman 1993; Schmidt et al. 2000; Verrelli and Eanes 2001; Sezgin et al. 2004). This contrast among functional classes of genetic markers suggests the action of spatially variable selection at particular polymorphic loci (e.g., McDonald 1994).

At the phenotypic level, there is also substantial evidence for nonrandom patterns of variation with latitudinal origin of *D. melanogaster* populations across continents. For example, traits such as body size (James et al. 1997; Gockel et al. 2001; Hoffmann et al. 2001), egg size and ovariole number (Azevedo et al. 1996), cold resistance (Hoffmann et al. 2002), heat resistance (Hoffmann et al. 2002; Frydenberg et al. 2003), ethanol tolerance (Cohan and Graf 1985), and desiccation resistance (Hoffmann and Parsons 1989) also exhibit latitudinal clines. Data from these and related studies are generally interpreted as evidence for adaptation to climatic variation. The evolutionary processes that generate such clines are dependent on the population dynamics of *D. melanogaster* in the wild; however, the basic ecology of this species is poorly understood.

Drosophila melanogaster is native to tropical Africa and has established itself in the New World in the last several hundred years (David and Capy 1988). Four lines of evidence support the hypothesis that gene flow in *D. melanogaster* is of sufficient magnitude to preclude genetic structure or local adaptation across the latitudinal gradient in the eastern United States. First, allele frequencies for presumably neutral genetic markers are geographically uniform (e.g., Hale and Singh 1991; Berry and Kreitman 1993). Second, release-recapture studies involving laboratory-reared mutants indicated dispersal of up to 10 km over a short time scale (Coyne and Milstead 1987). Third, passive transport via agricultural products is likely. Finally, the species is tropical in origin and is not thought to possess any mechanism associated with population persistence in temperate regions. Thus, populations in areas characterized by a strong degree of seasonality would most likely have to be reestablished from refugia or by human-mediated transport.

However, there is substantial evidence that *D. melanogaster* can overwinter as an adult in temperate habitats (Izquierdo 1991; Mitrovski and Hoffmann 2001; Boulétreau-Merle and Fouillet 2002). Temperate populations, rather than being solely recolonized from refugia or transport, may be temporally persistent by means of overwintering mechanisms. Although the actual mechanism has not been unambiguously identified, *D. melanogaster* can express a form of reproductive diapause (Saunders et al. 1989), similar to that of temperate endemics in the same genus (e.g., Lumme and Laa-

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kovara 1983; Kimura 1988). The incidence of diapause expression was shown to be greater in a population from Ontario, Canada, than in a population from Georgia, United States, consistent with the hypothesis that diapause expression is related to overwintering and population persistence (Williams and Sokolowski 1993). The expression of reproductive diapause in adult *D. melanogaster* is associated with decreased rates of age-specific mortality, increased life span, and increased resistance to multiple forms of environmental stress (Tatar et al. 2001). The ability to overwinter is strongly influenced by reproductive schedules as well as the timing of egg production and oviposition (e.g., Mitrovski and Hoffmann 2001; Boulétreau-Merle and Fouillet 2002; Hoffmann et al. 2003). Adaptation to temperate environments in this tropical insect appears to involve a suite of life-history traits related to stress resistance, patterns of reproduction, and correlated traits. However, the impact of genetic variance for diapause expression on these traits and its potential role in adaptation to temperate environments has not been addressed.

Here, we examine geographic variation for diapause incidence in natural *D. melanogaster* populations. We hypothesized that, if diapause is related to overwintering ability, the proportion of lines that express this trait would exhibit a latitudinal cline in frequency. We were particularly interested in evaluating whether lines that express diapause and those that do not are constitutively distinct for other traits that covary with latitude (e.g., stress resistance and lipid content). Given the established link between exposure to a temperate environment and reproductive dynamics, we also examined geographic variation in ovariole number and its relationship to fecundity as well as geographic variation in reproductive patterns over the life span.

Materials and Methods

Phenotype Determination

Twelve populations of *D. melanogaster* were collected from fruit orchards along the latitudinal gradient in the eastern United States by a combination of baiting and sweeping with aerial nets. Gravid females were immediately sorted into isofemale lines in the field; once the resulting progeny eclosed, lines were typed to species. Approximately 150 isofemale *D. melanogaster* lines were collected from each locale. Populations sampled were: Bowdoinham, Maine (44.03°N, 73.20°W); Whiting, Vermont (43.86°N, 73.20°W); Concord, Massachusetts (42.46°N, 71.36°W); Middlefield, Connecticut (41.52°N, 72.71°W); Lancaster, Pennsylvania (40.04°N, 76.30°W); Churchville, Maryland (39.44°N, 76.62°W); Richmond, Virginia (37.53°N, 77.47°W); Smithfield, North Carolina (35.51°N, 78.34°W); Macon, Georgia (32.84°N, 83.66°W); Jacksonville, Florida (30.33°N, 81.66°W); Merritt Island, Florida (28.31°N, 80.66°W); and Homestead, Florida (25.46°N, 80.45°W). Long-term maintenance of all populations was done at 17°C, 12:12 light:dark photoperiod, with a generation time of approximately 35 days. All life-history analyses were conducted within 24 months of collection. Prior to the initiation of an experiment, selected lines were transferred to a culture regime of 25°C under the same photoperiod for five generations. Sixty isofemale lines were randomly selected from each population for analysis of diapause incidence; diapause phenotype was determined using standard methodologies (Saunders et al. 1989; Williams and Sokolowski 1993; Tatar et al. 2001). Multiple virgin females from each of the 720 lines were collected in the same generation within 2 h posteclosion and placed on standard cornmeal medium at 12°C under a photoperiod of 10:14 light:dark. Four weeks later, flies from each line were dissected in a phosphate buffered saline (PBS) solution and their ovaries examined under a dissecting microscope. The most advanced oocyte from each set of ovaries was determined according to the developmental stages of King (1970); a line was scored as diapausing if the most advanced oocyte among three females was previtellogenic (stage 7 or less). Data were analyzed by nominal, logistic regression using JMP v. 4.04 (SAS Institute, Cary, NC).

Patterns of Inheritance for Diapause Phenotype

Approximately 100 inbred lines from each of the Whiting, Vermont, and Homestead, Florida, populations were created by 25 generations of full-sib mating in 1999–2000. Each line was thoroughly characterized for diapause phenotype according to the previously described method at multiple points in time. A given line was considered a diapause (D) line if the most advanced oocyte was at stage 7 or less in $>95\%$ of individuals in the assay; similarly, a line was considered nondiapause (ND) if multiple mature oocytes (stage 14) were present in $>95\%$ of individuals examined. While approximately 75% of the inbred lines could be clearly characterized as either D or ND, the remainder was seemingly intermediate (I) in phenotype. Of the lines characterized, 33 were classified as D, 27 as ND, and 19 as I. In the diapause assay, females from these intermediate (I) lines exhibited multiple vitellogenic oocytes of stages 8–12, but the ovaries rarely contained a mature (stage 14) follicle. The mean distribution of the most advanced oocyte in these I lines was as follows: 50.1% at stages 8–9, 44.7% at stages 10–12, and 5.2% at stage 14 ($SD = 0.161$).

Five inbred lines of the D and ND phenotype from both populations were selected for F_1 hybrid and F_2 backcross analyses. All pairwise crosses were performed for phenotype and population and were done in both directions. For the F_1 D/ND hybrids, five independent crosses were performed between D and ND lines from the Vermont population, five crosses between D and ND lines within the Florida population, and 10 D/ND crosses between the Vermont and Florida populations (see Table 1 for line numbers and specific crosses performed). For each cross, 50 virgin males and 50 virgin females were collected from replicate bottle cultures of each line. These flies were used to establish 20 crosses between any two given lines; 10 replicate vials were established containing five virgin males from the D line and five virgin females from the ND line, and 10 replicate vials each contained five virgin males from the ND line and five virgin females for the D line. Females were allowed to oviposit for 24 h and adults were then purged. F_1 flies were collected within 2 h posteclosion, placed in the diapause assay, and dissected in PBS four weeks later.

This crossing scheme was repeated to collect virgin F_1 D/ND hybrids for backcrosses to each parental line. All F_1 flies

TABLE 1. Diapause phenotype determination in F_1 diapause/nondiapause (D/ND) hybrids. Data are pooled across all replicates.

Population	Cross	Phenotype		Prop. D
		D	ND	
Vermont × Vermont	$D_{v1} \times ND_{v1}$	98	4	0.96
	$D_{v2} \times ND_{v2}$	76	68	0.53
	$D_{v3} \times ND_{v3}$	177	8	0.96
	$D_{v4} \times ND_{v4}$	141	4	0.97
	$D_{v5} \times ND_{v5}$	86	0	1.00
Florida × Florida	$D_{F1} \times ND_{F1}$	85	3	0.97
	$D_{F2} \times ND_{F2}$	85	1	0.99
	$D_{F3} \times ND_{F3}$	68	3	0.96
	$D_{F4} \times ND_{F4}$	158	0	1.00
	$D_{F5} \times ND_{F5}$	145	4	0.97
Vermont × Florida	$D_{v1} \times ND_{F1}$	54	0	1.00
	$D_{v2} \times ND_{F2}$	72	5	0.94
	$D_{v3} \times ND_{F3}$	84	10	0.89
	$D_{v4} \times ND_{F4}$	58	4	0.94
	$D_{v5} \times ND_{F5}$	67	0	1.00
Florida × Vermont	$D_{F1} \times ND_{v1}$	47	1	0.98
	$D_{F2} \times ND_{v2}$	86	0	1.00
	$D_{F3} \times ND_{v3}$	38	0	1.00
	$D_{F4} \times ND_{v4}$	91	0	1.00
	$D_{F5} \times ND_{v5}$	70	0	1.00

resulting from replicates of a specific cross were combined and then randomly assigned for F_2 backcrosses. Ten crosses were done within the Vermont population (10 line crosses, each done in both directions), 10 crosses within the Florida population, and 20 crosses between the Vermont and Florida populations (see Table 2 for line numbers and specific crosses). For each cross type, 10 replicate vials containing five virgin males and five virgin females were established. Adults were purged after 24 h and F_2 flies phenotyped for diapause as above.

Chromosome Introgressions

Chromosome substitutions were performed as an additional preliminary analysis of patterns of inheritance for diapause phenotype. One D (VT52i) and one ND (VT46i) line were selected; these lines were karyotyped to ensure that no chromosome was polymorphic for a common inversion and thus might reflect a balanced lethal system. Chromosomes were selected from the same locale so as not to introduce effects of variation for other traits that covary with latitude. Two additional lines were included: Canton-S and the isogenic line *6326* (Bloomington Stock Center; Hoskins et al. 2001). Canton-S is the line in which reproductive diapause in *D. melanogaster* was originally described (Saunders et al. 1989) and it tests as D in our assay. In the diapause assays we conducted, ovaries from the *6326* stock were also nonvitellogenic (see Results). Inbred lines in a white-eyed (w*) background were established by crossing males from each of the inbred lines with $w{*}T(2;3)ap^{Xa}/CyO{:}TM3$ females (Bloomington stock 2475) and recovering $w{*}CyO/+_{2i};TM3/+_{3i}$ (or $w{*}T(2;3)ap^{Xa}/+_{2i};\ +_{3i}$) male progeny, which were then backcrossed into the $w{*}T(2;3)ap^{Xa}/CyO;TM3$ translocation line. Male and female $w{*}CyO/+_{2i};TM3/+_{3i}$ sib progeny were intercrossed and $w{*}\ +_{2i};\ +_{3i}$ lines established. In the chromosome exchanges $w{*}\ +_{2i};\ +_{3i}$ males were crossed with $w{*}T(2;3)ap^{Xa}/CyO{:}TM3$ stock females and the $w{*}CyO/+_{2i};TM3/+_{3i}$ male progeny crossed with $w{*}CyO/Tft;+_{3j}/+_{3j}$ females for substituting the *i*th second chromosome into the *j*th third background and with $w{*}+_{2j}/+_{2j};TM3/Dr$ for the *j*th second into the *i*th third. From this cross male progeny of genotype $w{*}CyO/+_{i2};TM3/+_{j3}$ were again crossed with $w{*}CyO/Tft;+_{3j}/+_{3j}$ or $w{*}+_{2j}/+_{2j};TM3/Dr$ females and the $w{*}CyO/+_{2i};+_{3j}/+_{3j}$ or $w{*}+_{j2}/+_{j2};TM3/+_{i3}$ male and female sibs collected to finalize the respective substitutions. Not all homozygous combinations were viable. Each substitution line was maintained in replicate bottle cultures. For diapause phenotyping, adults from each line were transferred to five replicate culture bottles; females were allowed to oviposit for 24 h and were then transferred to new bottles. Progeny were collected within 2 h posteclosion across all replicates and phenotyped for diapause as previously described.

Starvation Resistance

From the Vermont and Florida inbred line populations, 10 D, 10 ND, and 10 I lines were selected and maintained in replicate cultures for two generations. For each population by genotype, five males and five females from each of the 10 inbred lines were randomly chosen and used to create five independent F_1 heterozygous lines. Reciprocal crosses were also performed to evaluate maternal effects. For example, in the Vermont inbred population males of line D_1 were crossed to females of line D_2, and males of line D_2 crossed to females of line D_1. These crosses were repeated for lines D_3–D_{10}, lines ND_1–ND_{10}, and lines I_1–I_{10}, and were replicated for lines of each phenotype in the Florida inbred population. Each specific cross was repeated 10 times to generate replicate vial cultures. Virgin females and males from each F_1 cross were collected every 8 h over a 24-h window: females were then either mated with males from the same cross for a period of 5 days (M) or maintained as virgins for the same time period (V). Groups of flies were then randomly assigned to one of three dietary treatments. In the starvation treatment (S), flies were held in sealed vials with no food but 5 ml of water in cotton. Mortality was recorded every 6 h until all flies were dead. The partial-starvation treatment (PS) was identical to the above but flies were starved for a period of 60 h and then frozen for later analysis of ovarian development; each female was dissected in PBS and the number of terminal stage 14 eggs counted. In the control treatment (C) flies were placed in vials containing standard cornmeal medium; after 60 h individuals in this treatment were also frozen for dissection as in the PS treatment. For each combination of population (Florida or Vermont) × genotype (D, ND, or I) × cross-type (e.g., male of line 1 crossed with female of line 2 or vice versa) × mating status (M or V) × treatment (S, PS, C) three replicates of six flies each were used. Throughout, all flies were maintained at 25°C and 12:12 light:dark and all experimental lines were analyzed simultaneously. At no time were flies exposed to environmental conditions that would induce diapause; thus, the experiment was designed to evaluate constitutive differences in survivorship between D and ND lines and not the effects of diapause expression per se. Survivorship data were analyzed with a nested ANOVA; data for the number of stage 14 eggs was analyzed with a nested ANCOVA with ovariole number as the covariate.

TABLE 2. Diapause phenotype determination in F_2 backcrosses. D, diapause; ND, nondiapause.

		Phenotype		
Population	Cross	D	ND	Prop. D
Vermont × Vermont	$D_{v1}/ND_{v1} \times D_{v1}$	215	3	0.99
	$D_{v1}/ND_{v1} \times ND_{v1}$	160	78	0.67
	$D_{v2}/ND_{v2} \times D_{v2}$	203	13	0.94
	$D_{v2}/ND_{v2} \times ND_{v2}$	65	104	0.38
	$D_{v3}/ND_{v3} \times D_{v3}$	171	9	0.95
	$D_{v3}/ND_{v3} \times ND_{v3}$	121	68	0.64
	$D_{v4}/ND_{v4} \times D_{v4}$	179	5	0.97
	$D_{v4}/ND_{v4} \times ND_{v4}$	93	70	0.57
	$D_{v5}/ND_{v5} \times D_{v5}$	187	0	1.00
	$D_{v5}/ND_{v5} \times ND_{v5}$	109	50	0.69
Florida × Florida	$D_{F1}/ND_{F1} \times D_{F1}$	156	0	1.00
	$D_{F1}/ND_{F1} \times ND_{F1}$	140	14	0.91
	$D_{F2}/ND_{F2} \times D_{F2}$	77	2	0.97
	$D_{F2}/ND_{F2} \times ND_{F2}$	81	43	0.65
	$D_{F3}/ND_{F3} \times D_{F3}$	137	6	0.96
	$D_{F3}/ND_{F3} \times ND_{F3}$	87	45	0.66
	$D_{F4}/ND_{F4} \times D_{F4}$	499	10	0.98
	$D_{F4}/ND_{F4} \times ND_{F4}$	151	103	0.59
	$D_{F5}/ND_{F5} \times D_{F5}$	145	4	0.97
	$D_{F5}/ND_{F5} \times ND_{F5}$	149	40	0.79
Vermont × Florida	$D_{v1}/ND_{F1} \times D_{v1}$	49	0	1.00
	$D_{v1}/ND_{F1} \times ND_{F1}$	19	41	0.32
	$D_{v2}/ND_{F2} \times D_{v2}$	62	4	0.94
	$D_{v2}/ND_{F2} \times ND_{F2}$	51	6	0.89
	$D_{v3}/ND_{F3} \times D_{v3}$	53	0	1.00
	$D_{v3}/ND_{F3} \times ND_{F3}$	51	15	0.77
	$D_{v4}/ND_{F4} \times D_{v4}$	105	12	0.90
	$D_{v4}/ND_{F4} \times ND_{F4}$	60	33	0.65
	$D_{v5}/ND_{F5} \times D_{v5}$	94	3	0.97
	$D_{v5}/ND_{F5} \times ND_{F5}$	54	45	0.55
Florida × Vermont	$D_{F1}/ND_{v1} \times D_{F1}$	41	4	0.91
	$D_{F1}/ND_{v1} \times ND_{v1}$	12	57	0.17
	$D_{F2}/ND_{v2} \times D_{F2}$	22	2	0.92
	$D_{F2}/ND_{v2} \times ND_{v2}$	12	31	0.28
	$D_{F3}/ND_{v3} \times D_{F3}$	18	16	0.53
	$D_{F3}/ND_{v3} \times ND_{v3}$	3	34	0.08
	$D_{F4}/ND_{v4} \times D_{F4}$	42	4	0.91
	$D_{F4}/ND_{v4} \times ND_{v4}$	40	35	0.53
	$D_{F5}/ND_{v5} \times D_{v5}$	59	2	0.97
	$D_{F5}/ND_{v5} \times ND_{v5}$	29	33	0.47

Triglyceride Content

The crossing scheme outlined above was repeated to measure lipid content of females. Three F_1 hybrid genotypes were recreated for the D and ND phenotypes from both the Vermont and Homestead, Florida, inbred populations. Progeny from each cross were collected, pooled across replicates, and randomly assigned to a treatment combination. For the mating status treatment, flies were either mated (M) or kept as virgins (V) for a period of 5 days. Subsequently, these experimental flies were either starved for a period of 3 days (S) or kept on standard medium for the same time period (C). All analyses were conducted simultaneously and flies were then frozen at −80°C until analysis. For each combination of predictor variables (population, phenotype, cross type), triglyceride content was determined for four replicate groups of three females according to a methodology modified from Clark and Keith (1988). Flies were homogenized on ice using a grinding rotor in 1 ml of phosphate buffer (0.025 M KH_2PO_4, pH 7.4). Samples were centrifuged at 1200 rpm for 2 min and the supernatant removed. Homogenates were stored at −80°C and briefly vortexed directly before use. The colorimetric assays were measured in a Beckman (Fullerton, CA) DU 640 spectrophotometer. Triglyceride content was determined using Sigma (St. Louis, MO) Kit 336-20, with three triglyceride (Sigma T 2522) standard dilutions (250 μg/ml, 500 μg/ml, and 1000 μg/ml). Each triglyceride assay contained 25 μl of sample (or standard) and 0.5 ml of reagent. Assays were staggered and incubated at 37°C for 4 h (all assays were completed within a 36-h period) and read at 500 nm. Dry mass was used to standardize all triglyceride measures. Frozen flies were placed in a 65°C oven for 24 h and weighed in a Cahn (C-32) microbalance. Triglyceride amount was log transformed and analyzed with a nested ANCOVA with log (mass) as the covariate. Results were qualitatively identical to those obtained by analyzing the residuals of an initial regression of triglyceride content on dry mass.

Ovariole Number

Two northern (MA and VT) and two southern (Merritt Island and Homestead, FL) populations were examined for

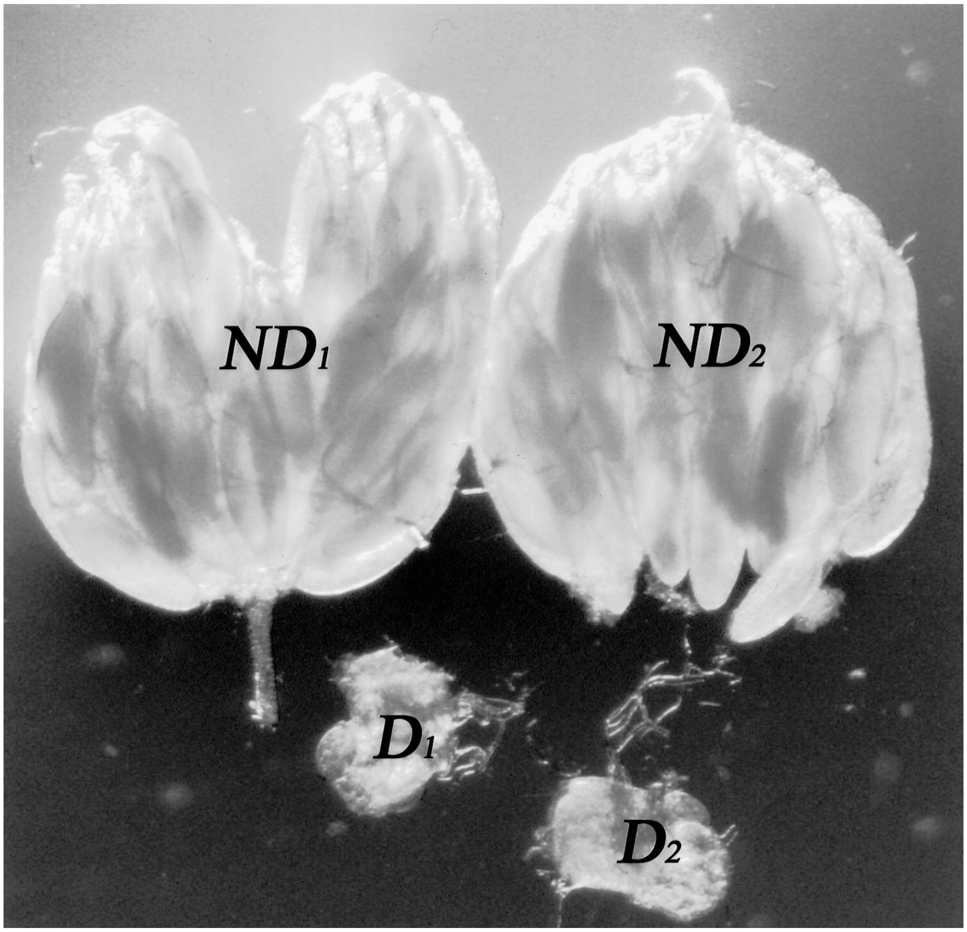

FIG. 1. The phenotype of reproductive diapause in *Drosophila melanogaster*. The two sets of ovaries are from a diapause (D) and nondiapause (ND) line from the Bowdoinham, Maine, collection. Females were placed in the diapause assay, ovaries dissected four weeks later, and visualized at 50X magnification.

ovariole number. Forty isofemale lines from each population were randomly chosen and analyzed at the same time; these lines were the same as those used in the fecundity analysis (see below). All lines were reared in three replicate cultures at 25°C and 12:12 L:D at relatively constant density (60–80 eggs/vial) for two generations prior to experimental initiation. Five virgin females were randomly collected from each line and maintained on standard cornmeal medium under the same temperature and photoperiod for 5 days. At this time, flies were transferred to 1.5-ml microfuge tubes and frozen at −80°C until dissection. Three flies were randomly selected and their ovaries dissected in PBS; ovaries were then immersed in saturated potassium dichromate for 3 min before a final transfer to PBS (Wayne and Mackay 1998). Ovarioles were separated with minutens (Bioquip Products, Gardena, CA) and counted. Data were analyzed with a nested ANOVA.

Fecundity and Longevity

The same isofemale lines from the Vermont, Massachusetts, and Merritt Island and Homestead, Florida, populations were used to evaluate geographic variation in fecundity profiles over the life span. Virgin flies from all lines were collected over a 24-h period; for each line, one female and one male were placed in an 8-dram vial containing standard cornmeal medium with food color additive. Adult flies were transferred to new vials every day and eggs counted under a dissecting microscope. Dead males were replaced with males of the same age from the same line.

The two populations at the ends of the latitudinal gradient (VT and Homestead, FL) were used in a preliminary analysis of geographic variation in life span. Forty isofemale lines from each population were cultured at low density (60 ± 20 eggs/vial) for two generations. Virgin males and females were collected from all lines over a 24-h period. Additional males and females of the same cohort were maintained to replace experimental flies when a mortality event occurred. One male and one female from each line were placed in a vial with standard cornmeal medium. Flies were transferred to new vials each day and any mortality events recorded.

RESULTS

Geographic Variation in Diapause Incidence

An example of the phenotype of reproductive diapause in given in Figure 1. Both sets of ovaries are from individuals of different lines exposed to the diapause assay for a period

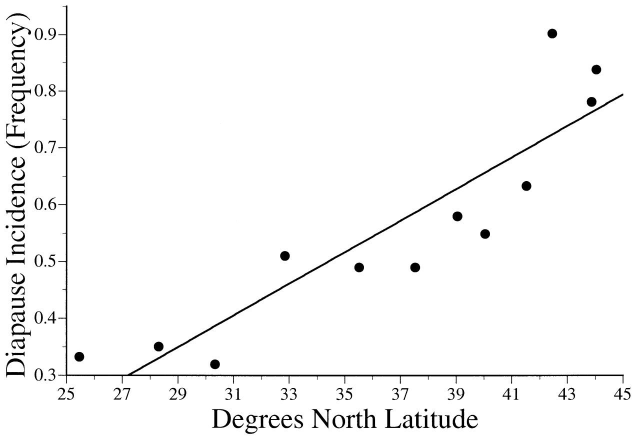

FIG. 2. Frequency of isofemale lines exhibiting diapause as a function of latitudinal origin of the source population. The general linear regression line is given ($r^2 = 0.8049$). In the nominal logistic regression, the odds of sampling a diapause line relative to a nondiapause line are significantly greater than 1.0 for the three northernmost populations (Walpole, ME; Whiting, VT; Concord, MA) and significantly less than 1.0 for the three southernmost populations (Jacksonville, FL; Merritt Island, FL; Homestead, FL).

TABLE 4. Phenotype determination for chromosome introgressions.

Genotype	Diapause frequency	*N*
w*;52;46	0.07	42
w*;52/CyO;46	0.00	38
w*;46;52	1.00	26
w*;46/CyO;52	0.97	37
w*;52;TM3/46	0.00	29
w*;46/CyOCanton-S	1.00	33
w*;46Canton-S	1.00	39
w*;Canton-S;46	0.00	41
w*;Canton-S;52	0.92	26
w*;6326;46	0.00	37
w*;6326;TM3/46	0.00	31
6326	1.00	40
Canton-S	0.90	36

of four weeks prior to dissection. The frequency of isofemale lines that expressed reproductive diapause was highly variable among populations, increasing from approximately 35% in Florida to 85% in northern New England (Fig. 2). The odds of an isofemale line from a northern (MA, VT, ME) population exhibiting diapause were 6.45 to 42.47 times higher than the baseline generated from all populations. Similarly, the odds of a line from a southern (Jacksonville, Merritt Island, and Homestead, FL) population exhibiting diapause were significantly less than 1.0. For the intervening populations (CT to GA), the odds of a given line expressing diapause in the assay were not significantly different from 1.0. Diapause incidence exhibited a significant latitudinal cline in frequency.

Inheritance Patterns for Diapause Phenotypes

All crosses within phenotypic classes (i.e., D × D or ND × ND) yielded the same phenotype as the parental lines (data not shown). In contrast, F_1 hybrids between D and ND parentals yielded progeny that expressed reproductive diapause in the laboratory assays (Table 1), suggesting the diapause phenotype was dominant to nondiapause. An exception was seen in one cross in the Vermont inbred population ($D_{V2} \times ND_{V2}$), which resulted in a F_1 D:ND ratio that was indistinguishable from 1:1 ($G = 0.508$, df = 1, $P > 0.40$).

Similarly, backcrosses of F_1 hybrids to the D parental line generally resulted in F_2 flies that expressed diapause (Table 2). As in the F_1 analysis, one cross deviated from this pattern: the backcross of the D_{F3}/ND_{V3} F_1 hybrid to the D_{F3} parental. The ratio of D:ND in this cross was equivalent to 1:1 ($G = 0.124$, df = 1, $P > 0.5$). If diapause segregates as a simple autosomal dominant, F_1 backcrosses to the ND parental line would be expected to yield an approximate D:ND ratio of 1:1 in the F_2 generation. This was not observed. However, there were significant effects of source population on the F_2 diapause phenotypic distributions (Table 3). In backcrosses within the Vermont and Florida inbred populations, the log odds of diapause/nondiapause were significantly elevated; in contrast, the odds of expressing diapause were significantly reduced in between-population crosses (VT × FL). Pooling across all VT × FL backcrosses to the ND parental line, the ratio of D:ND is equivalent to 1:1 (333 D, 299 ND; $G = 1.83$, df = 1, $P > 0.2$). For crosses within the Vermont and Florida populations, the D:ND ratio was approximately 2:1 (1156 D, 615 ND).

Chromosome Introgressions

Table 4 gives the frequency of diapause incidence in a series of lines that contained different combinations of second and third chromosomes; all lines had a common X chromosome. The chromosome introgressions indicated that the phenotype of a given introgressed line was determined by

TABLE 3. Nominal logistic regression analysis of the log odds (diapause/nondiapause) in F_2 backcrosses.

Term	Parameter estimate	SE	Chi square	*P*	Odds ratio
Intercept	1.587	0.055	818.32	0.0001	
Population (VT)	0.340	0.089	14.57	0.0001	1.974*
Population (FL)	0.783	0.098	63.92	0.0001	4.79*
Population (FL × VT)	−1.271	0.089	203.11	0.0001	0.0787*
Backcross to D parental	1.253	0.055	510.10	0.0001	12.246*
Population (VT) × backcross (D)	0.281	0.089	9.96	0.002	1.755*
Population (FL) × backcross (D)	0.208	0.098	4.52	0.034	1.517*
Population (FL × VT) × backcross (D)	−0.453	0.089	25.80	0.0001	0.404*

* The 95% confidence intervals do not include 1.0.

TABLE 5. Nested ANOVA for survivorship duration under starvation conditions.

Source	df	SS	*F*
Population	1	154.27	0.037
Phenotype	2	17,131.43	2.038
Mating status	1	56,762.03	201.976***
Line (phenotype, population)	25	108,160.18	2.223*
Maternal effects (line, phenotype, population)	29	57,514.55	7.057***
Population × phenotype	2	474.13	0.056
Phenotype × mating status	2	11,615.53	20.666***
Error	633	177,893.93	

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

the origin of the third chromosome. Lines possessing the VT46i (ND) third chromosome, either in a homozygous state or over the TM3 balancer, did not express diapause in the assay and contained multiple mature oocytes. In contrast, lines having the third chromosome derived from D sources (VT52i, Canton-S, or 6326) consistently arrested reproductive development and their ovaries were previtellogenic.

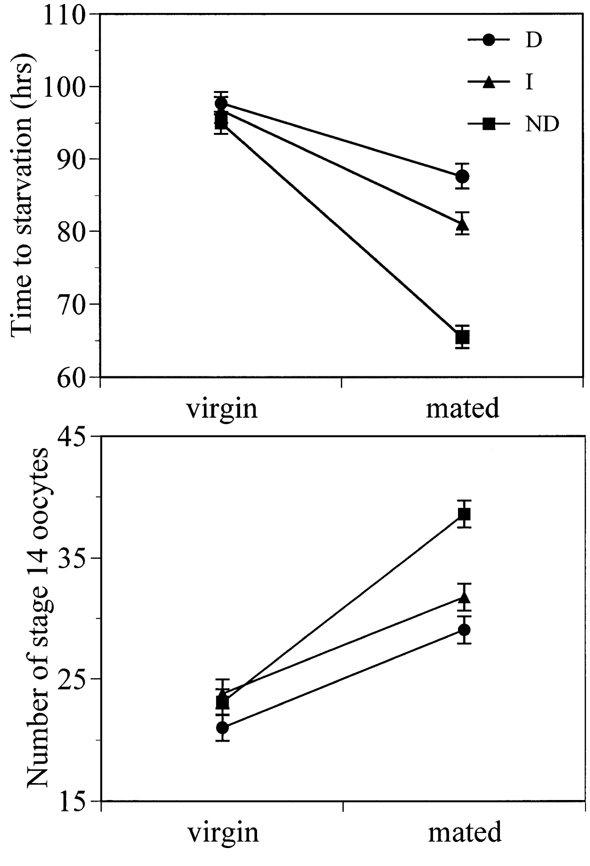

FIG. 3. Starvation resistance reaction norms (mean ± SE) for the diapause (D), intermediate (I), and nondiapause (ND) lines when assayed in the virginal and mated states. D and ND lines significantly differ in time to starvation in the mated condition only ($F_{1,633} = 51.757$, $P < 0.0001$). The bottom panel depicts the mean (± SE) number of terminal stage eggs in the partial starvation treatment for the three phenotypic classes when assayed as virgin and mated flies. D and ND lines are again distinct in the within model planned comparison when assayed in the mated condition only ($F_{1,817} = 39.896$, $P < 0.0001$).

Starvation Resistance

The starvation experiment was designed to evaluate whether genetic variance for diapause expression was associated with constitutive differences in survivorship under an ecologically relevant environmental stress. As expected, patterns of survivorship were highly variable among lines within populations and were also influenced by maternal source (Table 5). The diapause phenotype-by-mating status interaction term (Fig. 3a) was of primary interest due to the inherent difference in distribution of oocyte developmental stages in virgin and mated females. Mean time to starvation was equivalent for virgin ND and D line females (within model planned comparison: $F_{1,633} = 1.585$, $P < 0.208$). When assayed in the mated condition, however, D lines lived longer than did ND lines ($F_{1,633} = 51.757$, $P < 0.0001$). The I lines were intermediate with respect to survivorship.

Results from ovarian dissections of experimental flies in the PS and C treatments are given in Table 6. Of particular interest was the effect of the three-way interaction between treatment, mating status, and phenotype on the number of mature (stage 14) oocytes. In the control treatment, D and ND lines contained equivalent numbers of mature oocytes in both the virginal (planned comparison $F_{1,817} = 0.436$, $P < 0.509$) and mated conditions ($F_{1,817} = 0.0460$, $P < 0.830$). The results from the partial starvation treatment are depicted in Figure 3b. In the virginal state, D and ND lines contained statistically equivalent numbers of mature oocytes ($F_{1,817} = 1.412$, $P < 0.235$); in the mated condition, however, the number of stage 14 oocytes was significantly distinct between D and ND lines ($F_{1,817} = 39.90$, $P < 0.0001$).

Triglyceride Content

Standardized triglyceride content was variable within and between populations as well as between diapause phenotypes (Table 7). D lines contained significantly more triglyceride per body mass than did ND lines, and this pattern was consistent across populations. Although Vermont lines had more triglyceride than did lines from southern Florida, the effect of population was dependent on treatment. In the starvation treatment, in which triglyceride stores would be used and subsequently depleted, the two populations were equivalent ($F_{1,345} = 0.0222$, $P < 0.881$). In the control treatment, however, the populations were significantly distinct ($F_{1,345} = 21.821$, $P < 0.0001$).

TABLE 6. Nested ANCOVA for the number of stage 14 eggs in experimental flies.

Source	df	SS	*F*
Population	1	1,071.08	1.05
Phenotype	2	1,590.74	0.77
Treatment	1	6,450.65	73.49***
Mating status	1	70.28	0.8
Line (phenotype, population)	24	28,889.13	3.74**
Maternal effects (line, phenotype, population)	28	6,457.97	2.63***
Population × phenotype	2	2,709.76	1.37
Treatment × mating status	1	19,478.8	221.91***
Mating status × phenotype	2	2,568.31	14.63***
Treatment × phenotype	2	1,663.52	9.48***
Treatment × mating status × phenotype	2	1,048.82	5.97**
Ovarioles	1	1,187.81	13.53**
Error	817	71,715.21	

** $P < 0.005$; *** $P < 0.0001$.

Life-History Traits

Consistent with previous studies (e.g., Azevedo et al. 1996), ovariole number varied with geography (Fig. 4). Ovaries from flies of northern populations contained significantly (19%) more ovarioles than did ovaries from flies originating from southern locales ($F_{1,3} = 16.74$, $P < 0.03$). There was also a significant amount of variation between populations within regions ($F_{3,156} = 4.20$, $P < 0.01$) and among lines within populations and regions ($F_{156, 319} = 2.00$, $P < 0.001$). The relationship between ovariole number and fecundity was less straightforward, as ovariole number varied between northern and southern regions yet total lifetime fecundity was statistically equivalent. Furthermore, ovariole number was not a significant predictor of total fecundity (Table 8); this was also observed when populations were analyzed independently (not shown). In contrast to patterns of lifetime fecundity, per capita fecundity was higher for southern populations from days 2–22 but was higher for northern females from days 23–71. The difference in per capita fecundity between regions was non-randomly distributed over time by a runs test ($n_1 = 9$, $n_2 = 17$, four runs, $P < 0.005$; Sokal and Rohlf 1981).

The initial examination of geographic variation in life span demonstrated that longevity was higher for isofemale lines from Vermont than for lines from southern Florida (Fig. 5). The subsequent analysis of life span as well as fecundity of lines from two northern and two southern populations indicated that life-history profiles were distinct over the life span (Fig. 6). The fitness proxy was calculated for northern and southern regions as proportional reproduction multiplied by age-specific survivorship. These values were greater for the southern relative to northern populations early in life; this pattern reversed after approximately day 20, after which time the values were greater for northern samples. This general pattern was significant by a runs test ($n_1 = 25$, $n_2 = 59$, nine runs, $P < 0.005$).

DISCUSSION

Diapause is one of the most intensively studied adaptations to environmental heterogeneity in insects (e.g., Tauber et al. 1986). Although *D. melanogaster* was long thought to lack any seasonal response, individuals can persist as adults over the stressful winter period (e.g., Izquierdo 1991; Mitrovski and Hoffmann 2001; Boulétreau-Merle and Fouillet 2002). The cline in diapause incidence among populations across the east coast of the United States provides indirect yet compelling evidence that diapause expression is functionally related to overwintering mechanisms in this region. The results of Tatar et al. (2001) demonstrate that females held in diapause for up to 9 weeks exhibit negligible senescence over this time period. Thus, life-span extension and increased stress resistance via diapause expression could have a significant impact on population dynamics in natural habitats.

The temporal persistence of resident populations, by means of diapause expression or a functionally similar shift in reproductive allocation (e.g., Mitrovski and Hoffmann 2001;

TABLE 7. Nested ANCOVA for standardized triglyceride content in experimental flies.

Source	df	SS	*F*
Population	1	0.1438	11.61**
Phenotype	1	0.142	11.46**
Treatment	1	5.079	410.05***
Mating status	1	0.024	1.94
Line (phenotype, population)	8	0.802	8.09***
Maternal effects (line, phenotype, population)	12	0.152	1.025
Population × phenotype	1	0.0064	0.52
Population × treatment	1	0.1268	10.24*
Phenotype × treatment	1	0.0013	0.103
Mass	1	0.045	3.63
Error	345	4.273	

* $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

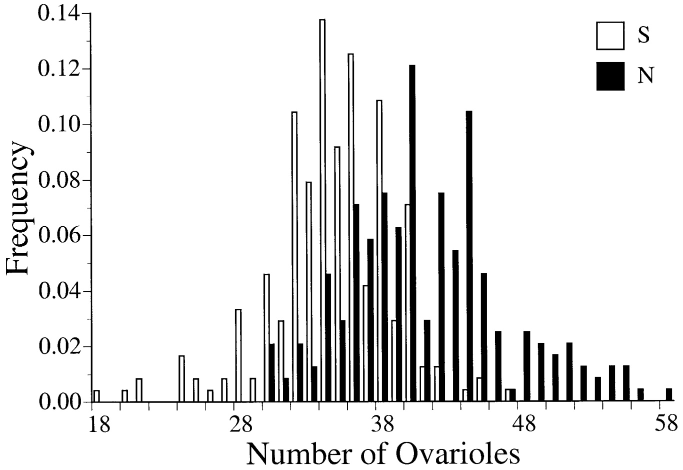

FIG. 4. Observed frequency of ovariole number counts for isofemale lines from northern (Whiting, VT, and Concord, MA, populations) and southern (Homestead, FL, and Merritt Island, FL, populations) regions. The range in ovariole number was 18–46 for the southern region (mean = 34.48) and 30–58 for the northern region (mean = 41.08).

Boulétreau-Merle and Fouillet 2002; Hoffmann et al. 2003), may have a significant impact on the population dynamics of *D. melanogaster* in temperate habitats. The hypothesis of widespread gene flow among populations that is supported by release-recapture dispersal studies of laboratory-reared mutants (Coyne and Milstead 1987), and the geographic distribution of mtDNA haplotypes (Hale and Singh 1991) contrasts with the vision of *D. melanogaster* population structure proposed by Wallace (1968). Both direct estimates of dispersal (Wallace 1970) and the spatial distribution of lethal alleles (Wallace 1966) suggest the potential for local differentiation that is predicated upon population persistence through the winter. Similarly, Ives (1945, 1970) consistently observed an eye color mutant (*cardinal-3*) in allelic screens involving a particular South Amherst, Massachusetts, population over a period of 30 years (1938–1968); this allele was never observed in any neighboring population during this time period, and the data were interpreted as evidence for temporal population continuity. Variable data regarding the magnitude of among-population gene flow in *D. melanogaster* may be due in part to distinct population and selective dynamics in urban centers versus rural areas (e.g., Kauer et al. 2003).

Unlike many other species of *Drosophila* that diapause as adults, in *D. melanogaster* the expression of diapause is highly variable among lines in a given population. The analysis of Williams and Sokolowski (1993) indicated that the diapause phenotype segregates as an autosomal recessive. The crosses involving inbred lines from two populations presented here indicated that diapause was dominant to nondiapause. The diapause phenotypic distribution in backcrosses to ND parental lines yielded variable results, and the data are not consistent with the action of a single Mendelian factor across all line crosses. The data for the chromosome introgression lines suggest that the genetic variance for diapause phenotype is associated with the third chromosome. These results were obtained using a small number of progenitor chromosomes, however, and thus the generality of this pattern cannot be addressed in the current study. Efforts are currently underway to evaluate the contribution of several candidate genes to variance in diapause expression as well as to generate a fine-scaled genetic map for this trait.

TABLE 8. Nested ANOVA for lifetime fecundity of two northern and two southern populations with population as a random factor.

Source	df	SS	*F*	*P*
Region	1	18,849.11	2.79	0.237
Population (region)	2	36,466.81	0.27	0.764
Ovariole number	1	67,881.79	1.00	0.318
Error	154	10,409,671		

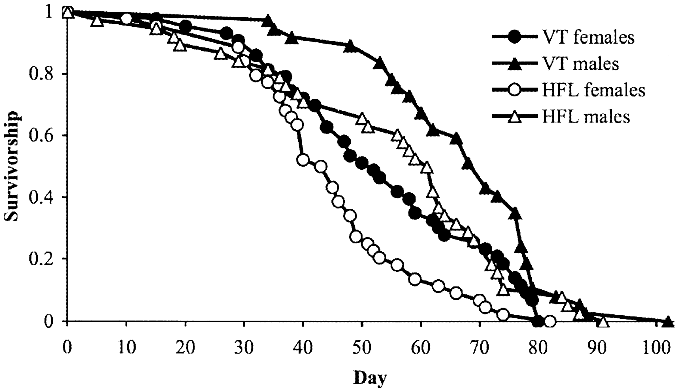

FIG. 5. Survivorship curves for males and females from the Whiting, Vermont, and Homestead, Florida, isofemale line populations. Both population ($F_{1,158} = 12.27$, $P < 0.001$) and sex ($F_{1,158} = 11.07$, $P < 0.01$) were significant predictors of log-transformed longevity.

Stress Resistance

The existence in natural populations of lines that express diapause and lines that do not highlights the need to evaluate the role of both variation in diapause expression (i.e., phe-

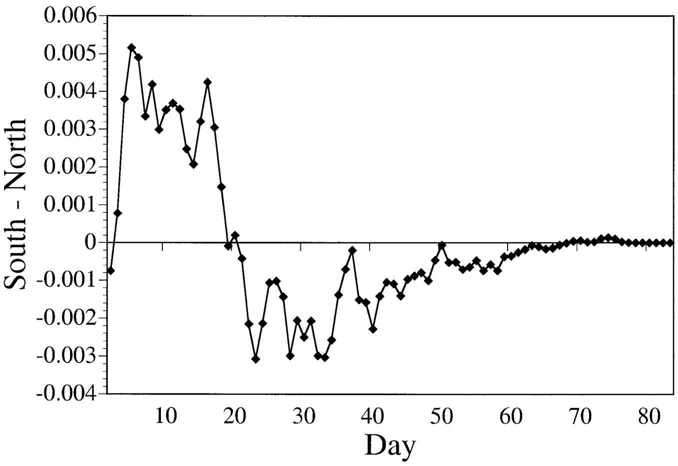

FIG. 6. Regional differences in proportional reproduction multiplied by age-specific survivorship as a function of time. The y-axis gives the difference between southern and northern populations; each datapoint is based on a sliding window of three-day means.

notype) and the ability to express diapause (i.e., genotype) on suites of life-history traits that may be involved in adaptation to environmental heterogeneity across this latitudinal gradient. Reproductive diapause is closely linked to vitellogenesis and the allocation of high-energy compounds such as lipids into reproduction. In other drosophilid species, triglyceride and lipid content are critical to overwintering ability and covary with diapause expression (e.g., Kimura 1988; Ohtsu et al. 1992). We have only assayed triglyceride content here, but lipid and triglyceride content are highly correlated in *D. melanogaster* (Clark and Gellman 1985). Lipid content is also a significant predictor of starvation resistance (e.g., Chippindale et al. 1993, 1996).

When assayed as virgins, females from D and ND lines exhibited equivalent patterns of survivorship under starvation conditions. However, a striking difference was observed when flies were mated: D lines survived an average of 32% longer than did ND lines. Two lines of evidence suggest that this difference in starvation tolerance is related to the differential availability of lipid-related compounds in D and ND lines. First, ovarian dissections of flies exposed to a nonlethal bout of starvation demonstrated distinct numbers of mature oocytes in the ovaries of ND and D females. This was not observed under control conditions, indicating a diapause phenotype-specific response to the stress. Soller et al. (1999) demonstrated that early vitellogenic stages in the *Drosophila* ovary may be actively resorbed, causing a two-fold increase in yolk protein concentration in the hemolymph. Thus, the observed difference in number of mature follicles between diapause phenotypes may reflect differential resorption of early-stage oocytes, although this was not directly evaluated. The equivalence of diapause phenotypes when tested for starvation resistance as virgins is unsurprising; virgin females have higher ecdysteroid titers than do mated females, which suppresses vitellogenesis (Harshman et al. 1999). Virgin females therefore have fewer early-stage oocytes that can be actively resorbed (Soller et al. 1999). Second, constitutive triglyceride content differed between D and ND lines, with D lines having more triglyceride per body mass.

Life Histories

Consistent with the hypothesis that selection pressures vary across the latitudinal gradient in the eastern United States, northern and southern populations are distinct for patterns of longevity, ovariole number, and age-specific reproduction. The isofemale and inbred lines used in the present study were cultured for approximately 25 generations prior to analysis. This is certainly sufficient time for adaptation to laboratory culture (e.g., Sgrò and Partridge 2000). However, all lines from all populations were cultured for the same duration, on the same food type and culture medium, and at the same temperature and photoperiod. Each trait investigated was analyzed simultaneously for all lines and populations. It is unlikely that the consistent differences among geographic regional sources were spuriously generated by adaptation to laboratory culture: the adaptation of lines from multiple populations to a common laboratory environment may have reduced rather than increased phenotypic variance among populations. This was not assessed, however, and it remains unknown how laboratory culture may have impacted the traits investigated. Geographic variation in life histories is currently being evaluated using recently collected isofemale lines from various sources.

Ovariole number is highly variable among natural populations (Lemeunier et al. 1986; Azevedo et al. 1996; Wayne et al. 1997) and exhibits genotype-by-environment interaction (Delpuech et al. 1995; Wayne et al. 1997; Wayne and Mackay 1998). This morphological trait is also associated with fitness, as ovariole number is correlated with both maximum daily fecundity (David 1970; Boulétreau-Merle et al. 1982) and among-population variation in body size (Lemeunier et al. 1986). No relationship between ovariole number and total fecundity was apparent in the present study. However, southern flies exhibited higher early versus late life fecundity when compared to lines from northern populations. This pattern of differential relative reproduction over time is correlated with differences in mortality rate and life span, as would be predicted (e.g., Rose 1984). Although the ovaries of southern females contained significantly fewer ovarioles, these flies laid more eggs per day in the first three weeks of life and an equivalent number of total eggs over the life span. Thus, reproductive flux through each ovariole and germarium was higher for southern relative to northern populations: ovaries from southern females may also have senesced at a faster rate. Epithelial stem cells in the *Drosophila* ovary do senesce, and functional loss is associated with subsequent reduction in fecundity (Margolis and Spradling 1995). In addition to impacting reproductive output during periods of maximum daily fecundity (e.g., David 1970), ovariole number may also be functionally related to temporal variation in reproduction (Carlson et al. 1998).

Coincident with the observed variation in life histories, diapause incidence significantly increased with latitude. Even in the absence of diapause expression or environmental cues, discernible phenotypic differences exist between genotypes that diapause and those that do not. Lines that express reproductive diapause have increased lipid content per body mass, distinct distributions of developmental stages in the ovaries following stress exposure, and enhanced resistance to starvation stress. Geographic variation in diapause incidence may result from selection on an associated trait, or variation for life-history profiles may reflect strong selection on diapause phenotype and/or overwintering ability. Currently, the genetic and phenotypic correlations of diapause are being assayed using sets of D and ND inbred lines from various populations.

Summary

Particular aspects of *Drosophila* life history vary predictably with latitude in the eastern United States, as on the Australian continent. Such patterns are consistent with climatic adaptation in this species and may reflect differential selection on the ability to overwinter. If, as indicated, diapause expression is variable within and between populations, diapause incidence varies among populations predictably with latitude, diapause and nondiapause genotypes are constitutively distinct for fitness-associated traits other than diapause expression, and life-history traits also vary predictably with geography, then we suggest a comprehensive treatment of life-history dynamics in natural *D. melanogaster* populations should include the diapause phenotype as an integral component. Additional field and laboratory experiments are being conducted to elucidate the mechanism of selection on diapause expression, its genetic and phenotypic correlates, and its role in adaptation of *D. melanogaster* populations to heterogeneous climatic environments.

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