JUVENILE HORMONE AS A REGULATOR OF THE TRADE-OFF BETWEEN REPRODUCTION AND LIFE SPAN IN *DROSOPHILA MELANOGASTER*

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Trade-offs between reproduction and life span are ubiquitous, but little is known about their underlying mechanisms. Here we combine treatment with the juvenile hormone analog (JHa) methoprene and experimental evolution in *Drosophila melanogaster* to study the potential role of juvenile hormone (JH) in mediating such trade-offs at both the physiological and evolutionary level. Exposure to JHa in the larval medium (and up to 24 h posteclosion) increased early life fecundity but reduced life span of normal (unselected) flies, supporting the physiological role of JH in mediating the trade-off. This effect was much smaller for life span, and not detectable for fecundity, in fly lines previously bred for 19 generations on a medium containing JHa. Furthermore, these selection lines lived longer than unselected controls even in the absence of JHa treatment, without a detectable reduction in early life fecundity. Thus, selection for resistance to JHa apparently induced some evolutionary changes in JH metabolism or signaling, which led to longer life span as a correlated response. This supports the hypothesis that JH may mediate evolution of longer life span, but—contrary to our expectation—this apparently does not need to trade-off with fecundity.

KEY WORDS: Aging, antagonistic pleiotropy, cost of reproduction, *Drosophila*, experimental evolution, juvenile hormone, life span, reproduction, trade-off.

In many organisms reproduction trades-off with survival (Williams 1966; Roff 1992). Such trade-offs may be observed at the physiological level (individuals that reproduce more live shorter), and at the evolutionary (genetic) level (evolution of higher reproductive effort is associated with reduced life span as a correlated response; Reznick 1985; Bell and Koufopanou 1986; Reznick 1992; Roff 1992; Stearns 1992). In the fruit fly (*Drosophila melanogaster*), reproductive factors that physiologically shorten life span include egg production (Partridge et al. 1987; Sgro and Partridge 1999), exposure to males (Partridge and Fowler 1990), and mating (Fowler and Partridge 1989; Chapman et al. 1995). Evolutionary trade-offs between fecundity and life span have been observed in numerous selection experiments (Rose 1984; Zwaan et al. 1995; Partridge et al. 1999; Stearns and Partridge 2001).

Little is known about the proximate mechanisms underlying these trade-offs (Leroi 2001; Barnes and Partridge 2003; Harshman and Zera 2007). A widely held view is that they are mediated through competitive resource allocation (Reznick 1985; Bell and Koufopanou 1986; van Noordwijk and de Jong 1986; Kirkwood and Rose 1991; de Jong and van Noordwijk 1992). Under this view, reproduction shortens life span because it withdraws limited resources that could otherwise be used for somatic maintenance and repair. However, a direct causal role for resource allocation has not been conclusively demonstrated (Rose and Bradley 1998; Barnes and Partridge 2003), and the observation that

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survival and reproduction can be experimentally decoupled in the nematode worm (*Caenorhabditis elegans*) and *D. melanogaster* is at odds with this notion (Hsin and Kenyon 1999; Arantes-Oliveira et al. 2002; Tu and Tatar 2003: Partridge et al. 2005). Alternatively, reproduction might accelerate damage-inducing processes independently of resource allocation, but this possibility has rarely been tested (Tatar and Carey 1995; Silbermann and Tatar 2000; Barnes and Partridge 2003). Thus specific genetic and physiological mechanisms mediating reproduction–survival trade-offs have so far rarely been identified (Rose and Bradley 1998; Leroi 2001; Barnes and Partridge 2003; Partridge et al. 2005; Harshman and Zera 2007). Furthermore, it is not clear how often trade-offs observed at the physiological and evolutionary level involve the same proximate mechanisms (Stearns 1989, 1992).

Given their central role in regulating physiology (Mangelsdorf et al. 1995; Schwartz et al. 2000), hormones are likely to be involved in modulating life-history trade-offs (Ketterson and Nolan 1992; Finch and Rose 1995; Dingle and Winchell 1997; Zera and Harshman 2001; Flatt and Kawecki 2004; Flatt et al. 2005; Harshman and Zera 2007). In insects, juvenile hormone (JH) is a major developmental and reproductive hormone, which affects multiple physiological processes by regulating gene expression in a variety of tissues (Nijhout 1994). Several lines of evidence suggest that JH stimulates reproduction at the expense of shorter life span (Flatt et al. 2005; Tu et al. 2006). In grasshoppers and butterflies, surgical removal of the corpora allata (glands producing JH) induces reproductive diapause and dramatically extends life span (Pener 1972; Herman and Tatar 2001; Tatar and Yin 2001). In *Drosophila*, mutants of the *Insulin-like Receptor* (*InR*) gene or the InR substrate *chico* are JH-deficient, exhibit ovarian arrest with nonvitellogenic oocytes, and are long-lived (Clancy et al. 2001; Tatar et al. 2001a; Tu et al. 2005). Similarly, in wild-type fruit flies undergoing reproductive diapause, JH synthesis is downregulated, ovarian development is arrested, and demographic senescence is reduced (Tatar and Yin 2001; Tatar et al. 2001b). When long-lived *InR* mutants or diapausing flies are treated with the JH analog (JHa) methoprene, survival is reduced and egg development is restored (Tatar et al. 2001a,b). However, because JH biosynthesis is also reduced in a sterile homozygous *InR* mutant genotype with normal longevity, JH deficiency might not be sufficient to extend life span (Tatar et al. 2001a). Furthermore, JHa treatment of sterile JH-deficient *chico* mutants cannot restore fecundity (Richard et al. 2005).

Despite much progress (Flatt et al. 2005; Tu et al. 2006), testing life-history effects of JH in small insects such as *Drosophila* remains challenging: (1) surgical removal of the corpora allata is difficult; (2) JHa and JH synthesis inhibitors can have pharmacological side effects (Wilson et al. 1983; Zera 2006); (3) null mutants of most genes involved in JH biosynthesis have not yet been isolated (Belles et al. 2005); (4) the molecular components of JH signal transduction remain largely unknown (Flatt and Kawecki 2004; Wilson 2004; Flatt et al. 2005; Tu et al. 2006); and (5) measuring JH biosynthesis and titers is difficult (Zera 2006). Consequently, how JH affects the trade-off between reproduction and life span is still poorly understood (Harshman and Zera 2007). In particular, it remains unclear whether JH modulates the trade-off between reproduction and survival in reproductively active, non-JH-deficient wild-type flies. This is the first issue we address in this paper.

Furthermore, the fact that hormonal manipulation (treatment with JH or JHa) and JH-deficient mutants tend to have antagonistic physiological effects on reproduction and survival does not automatically imply that changes in JH signaling or metabolism mediate an evolutionary trade-off between these fitness components (Flatt et al. 2005; Zera 2006). For that, two conditions must be satisfied. First, there must be additive genetic variation affecting aspects of JH metabolism or signaling (Flatt and Kawecki 2004; Zera 2006). Second, this variation must have antagonistic effects on reproduction and survival, in parallel to those caused by hormonal or genetic manipulations. It remains unknown whether these conditions are satisfied (cf. Flatt 2004a); this is the second issue we address in this paper.

Here we combined hormonal manipulation with experimental evolution to investigate a potential role of JH in the evolutionary trade-off between reproduction and survival in *D. melanogaster*. Aiming to induce evolutionary changes in JH metabolism or signaling, we exposed experimental populations to selection for resistance to deleterious effects of the JHa methoprene in the larval food medium. We hypothesized that such evolved changes in JH metabolism or signaling would have two effects on adult survival and fecundity. First, we expected the selected lines to become less sensitive than unselected control lines to the effects of JHa on life span and fecundity. Second, we predicted that selection for JHa resistance would lead to lower sensitivity of the flies to their own JH. If so, and if JH signaling indeed mediates the reproduction–survival trade-off, then the selected lines should show lower fecundity and longer life span than the control lines even without JHa treatment.

Material and Methods

METHOPRENE AS JHa

Juvenile hormone or its synthetic analog (JHa) methoprene can disrupt development and increase preadult mortality when applied throughout development or at a time when the hormone is not normally present (Wilson and Fabian 1986; Riddiford and Ashburner 1991). For that reason methoprene is used in insecticides; it is also widely used in insect physiology because it mimics JH action, but is better soluble, more potent, and more resistant to in vivo degradation than JH (Riddiford and Ashburner 1991; Wilson

2004; Zera and Zhao 2004). In particular, methoprene can act as a faithful JH agonist in a manner that is qualitatively identical to that of JH, both in vivo and in cell culture (Cherbas et al. 1989; Riddiford and Ashburner 1991; Wilson 2004; T. Flatt, unpubl. data). We thus used methoprene as an agent of selection.

Although JHa can be applied topically to adults, application via the food medium provides an easy and effective way of exposure. This method can specifically mimic JH activity and is efficient in treating a large number of flies (Riddiford and Ashburner 1991). Individuals exposed to dietary JHa continuously receive JHa through the gut by feeding, the cuticle by contact, and—because JHa produces a volatile vapor—the tracheal system by respiration (Wilson and Chaykin 1985; Wilson and Fabian 1986; Riddiford and Ashburner 1991; Wilson et al. 2003; T. G. Wilson, pers. comm.). Importantly, exposure of larvae to JHa in the food medium allowed us to impose selection on JH signaling or metabolism without directly selecting on adult survival, fecundity, or their responses to JHa.

The effects of dietary JHa may depend on culture density, and it is thus possible that feeding larvae degrade JHa, possibly by an enzyme in the saliva or the presence of gut bacteria (Wilson and Chaykin 1985). To avoid confounding effects of density on the effectiveness of JHa we therefore rigorously controlled larval densities in the selection experiment and all assays (see below). However, in a pilot experiment with the base stock, we found no evidence that the effects of JHa depend on larval density. When testing viability as a function of both JHa concentration (control: no JHa; treatment: 2.08 μl JHa per ml food) and egg density (100, 150, 200 eggs per bottle), we found that JHa induced about 25% egg-to-adult mortality (two-way ANOVA; $F_{1,26} = 1084.7$, $P < 0.0001$) irrespective of egg density ($F_{2,26} = 19.2$, $P = 0.79$). Furthermore, because JHa in the food medium is also taken up by contact/vapor and has been found to mimic the action of JH in several previous experiments (e.g., Wilson and Fabian 1986; Riddiford and Ashburner 1991; Wilson et al. 2003), it is unlikely that degradation can render dietary JHa fully ineffective.

Another potential caveat is that JHa (or its metabolites) in the larval diet might inhibit nutrient uptake or assimilation; differential effects of dietary JHa on adult life history in control versus selected flies could thus be due to differences in nutrient uptake or conversion efficiency rather than JH action per se. For example, malnutrition (starvation) decreases survival and fecundity, although dietary restriction increases survival, but lowers fecundity in *Drosophila* (Good and Tatar 2001; Tatar 2007). Thus, under both conditions, fecundity is reduced; however, our results were inconsistent with these alternatives (see below).

SELECTION LINES

We established eight JHa-resistant selection lines and eight JHa-susceptible control lines of *D. melanogaster*, all originating from an outbred base stock initiated with about 1000 flies in July 2000 and maintained in a population cage with a generation time of 2.5 weeks. The JHa-resistant lines were bred on a medium containing JHa. JHa (methoprene; Sigma-Aldrich; 1 μg/μl in 95% ethanol) was added to the still liquid, warm food medium to a final concentration of 1.04 μl per ml medium. This dosage was chosen based on a pilot dose–response experiment; it lowered egg-to-adult viability of the base stock by about 13% (Flatt 2004b). JHa-susceptible control lines were maintained under identical conditions, but were not exposed to JHa in the food medium.

For each of the 16 lines we established three culture bottles, with a controlled density of 200 eggs per bottle. In each generation, 15–16 days after egg laying, we randomly selected 30 females and 30 males from each bottle within a line. Adults from each bottle within a line were pooled for mating and females were allowed to oviposit overnight. The next day, we collected 600 eggs per replicate line and allocated them to a new set of three culture bottles, 200 eggs per bottle, to initiate the next generation. The 15–16 days generation time provided sufficient time for larval development and eclosion, allowing almost all viable adults to eclose (control: 99.8%; selection: 99.0%; T. Flatt, unpubl. data). The base stock and all experimental lines were maintained at 25°C, on a 12 h:12 h light:dark cycle, in bottles containing 25 ml of standard cornmeal-sugar-agar-yeast medium.

GENERAL ASSAY METHODS

We measured egg-to-adult viability, developmental time, body weight at eclosion, early fecundity, adult survival, and age-specific mortality of all JHa-resistant and JHa-susceptible lines under two test conditions: when raised on normal food medium and when raised on medium containing JHa. We used the same JHa concentration as that used to impose selection (1.04 μl/ml); this is important because, if the assay environment differs from the selection environment, results obtained from the assay may not correspond to the situation in the population under selection (Ackermann et al. 2001). Before carrying out life-history assays, all lines were kept for two generations without selection on normal food medium at controlled larval density to minimize parental effects. To obtain the individuals to be assayed, 200–300 adult flies from each line were placed into egg laying chambers containing plates with oviposition medium (a mixture of agar and orange juice) and females were allowed to oviposit overnight. The next day, we initiated 10 vials for each line, each vial with 50 eggs on 10 ml of normal food.

EGG-TO-ADULT VIABILITY

To test for a direct response to selection, after 7, 14, and 19 generations we measured egg-to-adult viability (proportion surviving) of all lines on normal medium and on medium containing JHa. To set up a viability assay, we placed 200–300 adult flies per replicate line

into egg laying chambers overnight. The next day, eggs from each line were allocated to 10 vials with normal food, and to 10 vials with food containing JHa (1.04 μl/ml), each vial with 50 eggs on 10 ml food ($n = 2$ selection regimes × 2 JHa conditions × 8 replicate lines × 10 vials = 320 vials). Vials were checked every 12 h for eclosing adults until all flies had emerged. We used repeated-measures multivariate analysis of variance (MANOVA) implemented in JMP IN 5.1. (SAS Institute; Sall et al. 2004) to determine the experiment-wide significance of main and interaction effects while controlling for within-treatment covariance (von Ende 2001). Thus, because viabilities within a given treatment might be correlated over time, among-treatment effects (selection regime, JHa treatment, JHa × regime) and within-treatment effects (time) are coordinately evaluated using exact F values based on Roy's greatest root (Harris 1985). Because sex ratio at eclosion was not affected by selection regime, JHa treatment, replicate line nested within regime, or interactions between these factors (analysis not shown), sexes were pooled for analysis of viability data.

LIFE SPAN AND MORTALITY

Adult survival and age-specific mortality were measured after 19 generations of selection. To set up the life span assay, we collected newly eclosed adult flies within a 24-h period. For each replicate selection and control line and each test condition, we established one 1-liter population cage ($n = 2$ selection regimes × 8 cages/lines × 2 JHa conditions = 32 cages). This factorial design allowed us to test for effects of selection regime, JHa treatment, and the JHa × regime interaction; however, we could not separately estimate the effects of replicate cage versus replicate line.

Each cage was initiated with 50 newly eclosed adults, mixed sex (see Tatar et al. 2001a,b for cage design). Dead flies were removed from cages and scored every two days, at which time fresh food was provided in a vial with 5 ml of standard cornmeal-sugar-agar-yeast medium. Cages were maintained at 25°C, on a 12 h:12 h light:dark cycle. Note that, irrespective of the larval medium and selection regime, flies were not exposed to JHa during adulthood (except for up to 24 h between eclosion and being collected for flies raised on JHa-containing medium).

Survival data were pooled across replicate cages within a treatment. From these data we constructed life tables by the extinct cohort method (Chiang 1984). Adult survival (fraction of flies alive, l_x) was calculated as N_x/N_0, where N_x is the number of flies alive at the beginning of each census interval and N_0 is the initial cohort size. Data were analyzed using Kaplan–Meier survival analysis implemented in JMP IN 5.1. (Sall et al. 2004); significant differences in survival between pairs of cohorts were tested by using the log-rank test (Parmar and Machin 1995).

To obtain additional insights into the pattern of mortality change, we estimated age-specific instantaneous mortality rate as $\ln(\mu_x) \approx \ln(-\ln[1 - D_x/N_x])$, where D_x is the number of dead flies in a given census interval (Elandt-Johnson and Johnson 1980). Because in many species, including *Drosophila*, mortality rates increase exponentially with age (Carey et al. 1992; Curtsinger et al. 1992), we fitted a standard model describing such a mortality trajectory to our data, namely the Gompertz model: $\mu_x = \lambda e^{\gamma x}$, where x is age, λ is baseline mortality or "frailty," and γ is the rate at which mortality increases as a function of age x (Elandt-Johnson and Johnson 1980). The intercept parameter λ (frailty) represents the individual susceptibility or "proneness" to death due to systems that degenerate progressively with age; the slope parameter γ is interpreted as the rate of aging, reflecting the progressive degeneration of somatic function within individuals. We fitted Gompertz parameters to each cohort using maximum likelihood estimation (MLE) implemented in WinModest (Pletcher 1999) and tested for differences in parameter values among pairs of cohorts using log-likelihood ratio tests. To test for effects of selection regime, JHa treatment, and JHa × regime on mortality we used proportional hazards analysis (Cox regression; Parmar and Machin 1995) implemented in JMP IN 5.1. Analyzing survival and mortality patterns separately for females and males did not affect the outcome of our analyses; similarly, proportional hazards analysis did not reveal a significant sex × JHa × regime interaction (analyses not shown). We therefore pooled survival and mortality data for both sexes.

EARLY FECUNDITY

For each population cage in the life span assay, we counted all eggs laid during the first five 48-h periods as estimates of early fecundity over the first 10 days after eclosion (5 × 32 = 160 vials). Age-specific daily fecundity was estimated as the average number of eggs laid per female per 48-h interval. When estimating fecundity, egg counts were averaged over all reproductive females alive in a given 48-h period. Data on age-specific fecundity were analyzed using repeated-measures MANOVA implemented in JMP IN 5.1. (Sall et al. 2004).

DEVELOPMENTAL TIME AND BODY WEIGHT AT ECLOSION

Because effects of selection and/or JHa treatment on reproduction and life span might be confounded by inadvertent effects on developmental time and body weight, we assayed these traits after 14 generations of selection in all lines, both on normal food and on food containing JHa. For both assays, 200–300 adult flies per line were placed into egg laying chambers overnight. The next day, eggs from each line were allocated to two vials with normal food, and to two vials with food containing JHa (1.04 μl/ml), each vial with 50 eggs on 10 ml food ($n = 2$ selection regimes × 2 JHa conditions × 8 replicate lines × 2 vials = 64 vials). Vials were checked for eclosing adults twice a day from day 7 after

egg laying. Average developmental time was calculated once all flies had eclosed. Within 12 h of emergence, flies were frozen, dried for three days at 80°C, and weighed individually on a Mettler MT5 balance to an accuracy of 0.001 mg. Data for both traits were analyzed with JMP IN 5.1. (Sall et al. 2004), using a nested mixed-effects ANOVA model:

$$X = \mu + A_i + B_j + AB_{ij} + C(A)_{k(i)} + BC(A)_{jk(i)} + \text{error},$$

where μ = mean, A = selection regime (fixed factor, two levels: selection, control), B = JHa treatment (fixed factor, two levels: JHa, no JHa), $C(A)$ = lines nested in selection regime (random factor, eight levels: eight independent replicate lines).

Results

EGG-TO-ADULT VIABILITY

JHa reduced egg-to-adult viability in unselected (JHa-susceptible) control flies, but not in selected (JHa-resistant) flies, suggesting that selected flies evolved significant levels of resistance to JHa (Fig. 1, Table 1; JHa × regime interaction, contrast between selected and control flies treated with JHa: exact $F_{1,28} = 6.02$, $P = 0.02$). Egg-to-adult viability of resistant flies treated with JHa increased from 63% in generation 7 to 71% in generation 19, whereas treatment of susceptible control flies with JHa decreased their viability on average by 19% (average of three assays). Selected flies assayed on medium without JHa did not have reduced egg-to-adult viability, indicating that JHa-resistant flies did not pay a detectable viability cost of resistance (Fig. 1; JHa × regime interaction, contrast between selected and control flies without JHa: exact $F_{1,28} = 1.86$, $P = 0.18$).

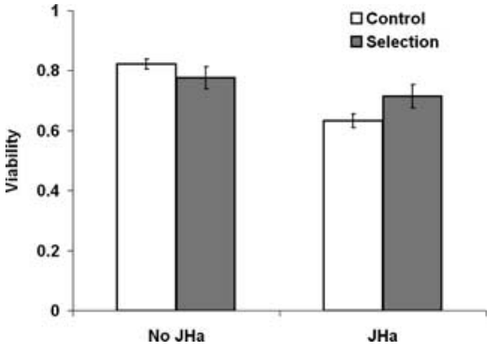

Figure 1. Egg-to-adult viability (proportion surviving) of selected lines and unselected control lines as a function of JHa treatment. Data shown are means ± standard errors (SE) of replicate lines within a selection regime, averaged across three viability assays performed after 7, 14, and 19 generations of selection. JHa treatment reduced egg-to-adult viability in unselected JHa-susceptible control flies, but not in selected flies that evolved resistance to JHa. Also see Table 1.

Table 1. Repeated-measures MANOVA for egg-to-adult viability (proportion egg-to-adult survival) measured after 7, 14, and 19 generations of selection. Also see Figure 1.

Source	Roy's greatest root	F	df_{num}	$df_{den.}$	P
Among treatments					
JHa treatment	1.03	28.70	1	28	<0.0001
Selection regime	0.02	0.59	1	28	0.44
JHa×regime	0.26	7.29	1	28	0.012
Within treatments					
Time	352.80	4762.80	2	27	<0.0001
Time×JHa	0.29	3.97	2	27	0.038
Time×regime	0.12	1.56	2	27	0.22
Time×JHa×regime	0.15	2.00	2	27	0.15

LIFE SPAN AND MORTALITY

Exposure to JHa during development strongly reduced subsequent adult survival and life expectancy in control flies, but to a much lesser extent in JHa-resistant flies, which had greater survival than control flies (Fig. 2A, Tables 2 and 3). Thus, JHa reduced the longevity of flies, but JHa-resistant flies evolved partial insensitivity to these life span shortening effects.

JHa-resistant flies also evolved significantly extended life span relative to the JHa-susceptible control flies in the absence of JHa (Fig. 2A, Tables 2 and 3). Thus, evolutionary changes in JH metabolism or signaling due to selection for improved JHa resistance caused life span extension in a normal environment. JHa treatment of long-lived JHa-resistant flies restored median life span to the level seen in untreated control flies (Fig. 2A, Tables 2 and 3; control flies without JHa: 44 days; long-lived JHa-resistant flies: without JHa: 46 days, with JHa: 44 days).

Gompertz and proportional hazards analyses of age-specific mortality confirmed that JHa shortens life span (Fig. 2B, Table 2; Cox regression, effect of JHa: likelihood-ratio $\chi^2 = 74.6$, $P < 0.0001$). JHa overall increased mortality early in life, but this effect diminished with age, either because the flies cleared off JHa (it was not present in the adult medium), or because the most susceptible individuals died first (Fig. 2B). JHa had different effects on mortality in unselected versus selected flies (Cox regression, JHa × regime: likelihood-ratio $\chi^2 = 19.4$, $P < 0.0001$). In JHa-susceptible control flies, JHa treatment significantly increased frailty (λ), the baseline susceptibility to death, but decreased the Gompertz slope parameter γ (Fig. 2B, Table 2). In JHa-resistant flies, JHa treatment did not affect mortality parameters, thus confirming that JHa-resistant flies evolved insensitivity to the life span shortening effects of JHa (Fig. 2B, Table 2). Furthermore, JHa-resistant and JHa-susceptible flies were genetically differentiated with respect to mortality parameters (Fig. 2B, Table 2;

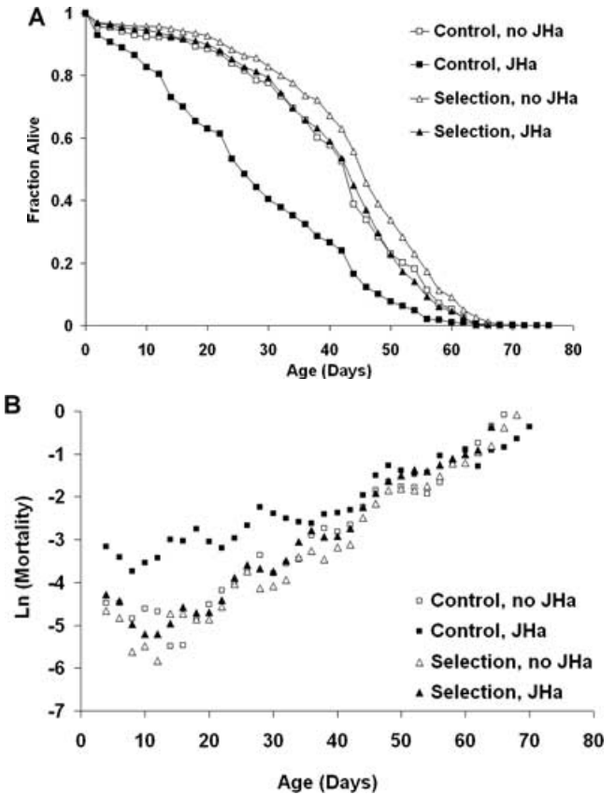

Figure 2. Adult survivorship and age-specific mortality rates of selected lines and unselected control lines as a function of JHa treatment. (A) Adult survivorship (fraction of flies alive, l_x), for both sexes pooled. JHa treatment strongly reduced survivorship of unselected JHa-susceptible control flies, but only moderately decreased survival of selected JHa-resistant flies. Treatment of JHa-resistant flies with JHa restored longevity to the level seen in unselected control flies not treated with JHa (compare solid triangles with open squares). As compared to unselected control flies, selected flies evolved increased adult survival and extended median life span. Together, these data suggest that JHa shortens life span. See Tables 2 and 3 for survival statistics. (B) Age-specific mortality rates (natural logarithm of μ_x), for both sexes pooled. For clarity, mortality rates were smoothed using running averages over three census intervals (six days). JHa treatment strongly increased frailty (λ) in unselected control flies, but not in selected JHa-resistant flies, suggesting that JH increases the baseline susceptibility of individuals to death. Selected flies evolved decreased frailty across age classes relative to unselected control flies. See Table 2 for mortality statistics.

Cox regression, selection regime: likelihood-ratio $\chi^2 = 74.4, P < 0.0001$). In the absence of JHa, JHa-resistant flies showed reduced frailty as compared to unselected control flies; in the presence of JHa, long-lived JHa-resistant flies exhibited reduced frailty, but an increased rate of aging (Fig. 2B, Table 2).

EARLY FECUNDITY

JHa treatment significantly increased age-specific fecundity over the first 10 days of adult life, thus confirming the well-known role of JH as a reproductive hormone (Fig. 3, Table 4). However, selection regime and the JHa × selection regime interaction did not affect fecundity (Table 4). Contrasts analysis confirmed that control flies and selected flies were not genetically differentiated in terms of early fecundity (Fig. 3; contrast, selected versus control flies, without JHa: exact $F_{1,28} = 0.11$, $P = 0.74$; with JHa: exact $F_{1,28} = 0.16$, $P = 0.69$). Although JHa significantly increased early fecundity of JHa-susceptible control flies (Fig. 3; contrast, exact $F_{1,28} = 5.08$, $P = 0.03$), fecundity of JHa-resistant flies was insensitive to treatment with JHa (contrast, exact $F_{1,28} = 0.48$, $P = 0.49$). These results also suggest that the life span shortening effects of JHa were likely to be physiological because the same dosage of JHa positively affected fecundity in control flies.

DEVELOPMENTAL TIME AND BODY WEIGHT AT ECLOSION

JHa treatment increased developmental time of flies on average by 16.8 h (6.7%) as compared to flies assayed on normal food medium, yet selection regime and the JHa × regime interaction had no effect on this trait (Fig. 4A, Table 5). JHa treatment reduced body weight at eclosion by 13.5% (approximately 0.03 mg); however, the selection regime and JHa × regime interaction did not affect weight (Fig. 4B, Table 6). Thus, the absence of correlated responses for both traits suggests that the prolonged life span and the insensitivity to effects of JHa on reproduction and life span observed in JHa-resistant flies is unlikely to be a consequence of selection on developmental time and weight. Similarly, because JHa treatment had similar effects on developmental time and weight at eclosion in JHa-susceptible control and JHa-resistant selected flies, flies in the two selection regimes were unlikely to differ in nutrient uptake or assimilation.

Discussion

Pleiotropic hormones are thought to be important regulators of life-history trade-offs (Tatar et al. 2003; Flatt and Kawecki 2004; Flatt et al. 2005; Harshman and Zera 2007). In *Drosophila* and other insects, JH has been proposed to stimulate reproduction at the expense of survival (Flatt et al. 2005; Tu et al. 2006). This makes JH a candidate target of natural selection on the trade-off between reproduction and survival; a mechanism that could mediate evolutionary shifts of the life history. Several aspects of our results provide support for this hypothesis.

First, our results confirm the physiological role of JH in stimulating reproduction and reducing survival (Flatt et al. 2005; Tu et al. 2006). We extend previous results by showing that this effect

Table 2. Survival and mortality statistics. Mortality parameters λ (frailty) and γ (rate of aging) were estimated from the Gompertz model using MLE. Parameters were compared among cohorts stratified by JHa treatment and selection regime (A vs. B; C vs. D; A vs. C; and B vs. D) using log-likelihood ratio tests. Shared superscripts denote nonsignificant comparisons; all significant results ($P < 0.001$) remained significant after Bonferroni correction for multiple comparisons. Starting values for the MLE procedure were $\lambda = 0.0001$ and $\gamma = 0.10$. Analyzing sexes separately did not change the results; sexes were thus pooled. Cohort size is the initial cohort size (total initial number of flies per treatment, pooled across replicate cages). Adult life expectancy (days) was estimated from eclosion; median life span is the age (in days) at which half the subjects have failed. Also see Figure 2.

Cohort	λ	γ	Cohort size	Median life span	Life expectancy
(A) Control, no JHa	0.002	0.076[a]	366	44	39.5
(B) Control, JHa	0.013	0.044	388	26	27.3
(C) Selection, no JHa	0.001[a]	0.085[a,b]	357	46	43.3
(D) Selection, JHa	0.002[a]	0.082[b]	348	44	40.0

Table 3. Log-rank tests for differences in adult survival (fraction of flies alive), stratified by JHa treatment and selection regime. All results remain significant after Bonferroni correction for multiple comparisons. Also see Figure 2

Comparison between cohorts	Effect	χ^2	P
A, B: Control (no JHa), Control (JHa)	Effect of JHa in Control	87.4	<0.0001
C, D: Selection (no JHa), Selection (JHa)	Effect of JHa in Selection	13.2	0.0003
A, C: Control (no JHa), Selection (no JHa)	Effect of Selection without JHa	12.4	0.0004
B, D: Control (JHa), Selection (JHa)	Effect of Selection with JHa	88.8	<0.0001

occurs in reproductively active, non-JH-deficient wild-type flies, and even if the JH treatment is limited to the larval stage and the first 24 h after adult eclosion. Supplementing larval food medium with the JHa methoprene increased fecundity of unselected JHa-sensitive flies, but reduced their life span, confirming the antagonistic physiological effect of JH on reproduction and survival (Flatt et al. 2005; Tu et al. 2006). Inspection of mortality rates suggested that the effect of JHa treatment on mortality was particularly strong within the first two to three weeks of adult life, and became progressively smaller at later ages. This is confirmed by the Gompertz model: JHa treatment significantly increased the Gompertz intercept parameter λ (frailty) of JHa-sensitive lines, but reduced their Gompertz slope parameter γ. However, this does not necessarily mean that JHa treatment slowed down the rate of aging. The simplest explanation for this pattern is that the effect of JHa simply wore off with age, as the flies cleared it out of their system (JHa was only added to the larval medium and not re-applied during adult life). This result is consistent with the

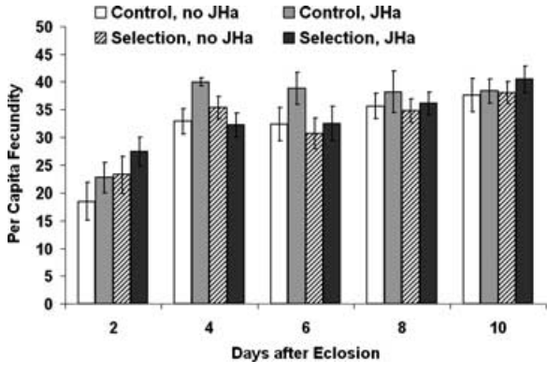

Figure 3. Early fecundity over the first 10 days posteclosion (average number of eggs laid per female per 48-h interval, ± SE) of selected lines and unselected control lines as a function of JHa treatment. JHa treatment significantly increased fecundity in unselected control flies, confirming the reproductive function of JH. In contrast, JHa had no effect on the reproductive output of selected JHa-resistant flies. See Table 4 for fecundity statistics.

Table 4. Repeated-measures MANOVA for fecundity (average number of eggs laid per female per 48-h interval) over the first 10 days posteclosion. Also see Figure 3.

Source	Roy's greatest root	F	df_{num}	$df_{den.}$	P
Among treatments					
JHa treatment	0.2	5.61	1	28	0.025
Selection regime	0.00008	0.002	1	28	0.96
JHa×regime	0.0096	0.27	1	28	0.61
Within treatments					
Time	3.92	25.54	4	25	<0.0001
Time×JHa	0.02	0.12	4	25	0.97
Time×regime	0.26	1.63	4	25	0.19
Time×JHa×regime	0.25	1.54	4	25	0.22

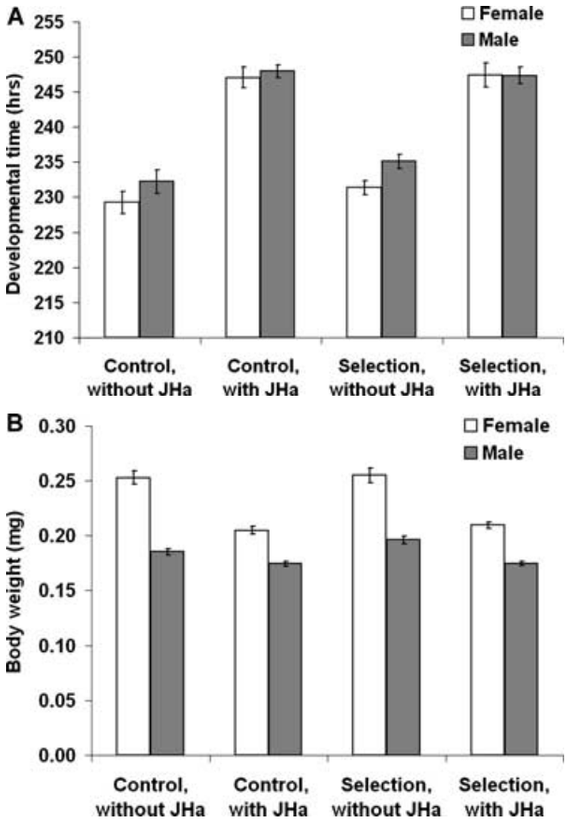

Figure 4. Developmental time (A, in hours) and body weight at eclosion (B, in mg) of selected and unselected control lines as a function of JHa treatment. Data shown are means ± standard errors (SE). JHa treatment prolonged developmental time and reduced weight at eclosion, both among selected and unselected control lines. However, the JHa × selection regime interaction was nonsignificant for both traits; thus neither trait showed a correlated response to selection. See Tables 5 and 6 for statistical analyses of developmental time and body weight data.

observation that removal of the corpora allata extends life span in butterflies mainly by reducing frailty, whereas JH treatment of allatectomized individuals increases frailty (Herman and Tatar 2001).

Second, we found that flies can evolve reduced sensitivity to the effects of JHa. Lines maintained on a JHa-containing medium evolved partial resistance to the adverse effects of JHa on egg-to-adult viability. As we had hypothesized, these JHa-resistant flies also became less sensitive to the physiological effects of JHa treatment on reproduction and life span. The effect of JHa treatment on adult survival was much smaller in JHa-resistant lines than in unselected JHa-sensitive lines. When developing on JHa-containing medium, JHa-resistant flies lived substantially longer than JHa-susceptible flies, and only slightly shorter than JHa-resistant flies bred without JHa. In contrast to JHa-sensitive lines, larval JHa treatment did not detectably increase fecundity of JHa-resistant lines, in line with our predictions. Thus, although we could not find statistical evidence for a correlated fecundity response to selection, JHa treatment seemed to promote fecundity in JHa-susceptible flies, but not in JHa-resistant flies. Our results demonstrate that the base population from which our selection lines were derived harbored heritable variation for the response to JHa. This variation not only allowed the selected lines to improve their egg-to-adult viability on a JHa-containing medium, but also led to reduced sensitivity to the effect of JHa on life span and reproduction. Importantly, these effects on reproduction and life span were not confounded by physiological effects of JHa on developmental time and weight at eclosion or by inadvertent selection on these traits: JHa treatment affected both traits similarly in control and selected flies, and neither trait showed a correlated response to selection.

Third, and most interestingly, as a correlated response JHa-resistant lines evolved lower adult mortality in the absence of JHa

Table 5. ANOVA for developmental time (hours). Because of the unbalanced nature of the data, Satterthwaite's approximation was used to construct approximate degrees of freedom and *F*-tests. Also see Figure 4A.

Source	F	df_{num}	$df_{den.}$	P
JHa treatment	242.24	1	14.07	<0.0001
Selection regime	1.98	1	5.52	0.21
JHa×regime	1.71	1	14.07	0.21
Replicate line (Regime)	3.60	14	5.50	0.07
JHa×Line (Regime)	1.95	14	14	0.11
Sex	15.25	1	14.30	0.002
Sex×JHa	4.27	1	14.13	0.06
Sex×Regime	0.01	1	14.30	0.92
Sex×JHa×Regime	0.38	1	14.13	0.55
Sex×Line (Regime)	0.45	14	14	0.93
Sex×JHa×Line (Regime)	1.00	14	62	0.46

Table 6. ANOVA for body weight at eclosion (mg). Because of the unbalanced nature of the data, Satterthwaite's approximation was used to construct approximate degrees of freedom and *F*-tests. Also see Figure 4B.

Source	F	df_{num}	$df_{den.}$	P
JHa treatment	72.24	1	14	<0.0001
Selection regime	0.93	1	14	0.35
JHa×regime	0.28	1	14	0.61
Replicate line (Regime)	1.26	14	18.84	0.31
JHa×Line (Regime)	6.29	14	14	<0.001
Sex	383.80	1	14	<0.0001
Sex×JHa	106.90	1	14	<0.0001
Sex×Regime	0.22	1	14	0.65
Sex×JHa×Regime	5.00	1	14	0.04
Sex×Line (Regime)	2.80	14	14	0.03
Sex×JHa×Line (Regime)	0.31	14	64	0.99

treatment (reduced frailty parameter of the Gompertz model). As a result, their life span in the absence of JHa was on average 3.8 days (9.6%) longer than that of unselected JHa-susceptible lines. We hypothesized that selection for JHa resistance would induce compensatory changes that would effectively reduce JH metabolism or signaling, with effects on life history resembling those of mild JH deficiency. The longer life span of the JHa-resistant flies is consistent with this hypothesis: extension of life span is typically observed in JH-deficient flies (Tatar and Yin 2001; Tatar et al. 2001a,b). However, JH-deficient flies also typically show impaired ovarian development or reduced fecundity (Tatar and Yin 2001; Tatar et al. 2001a,b; Flatt et al. 2005), which we did not observe in our JHa-resistant lines. Thus, our results support the notion that changes in JH metabolism or signaling may mediate evolutionary changes in life span, but they do not provide evidence that these changes would also mediate the evolutionary trade-off with fecundity.

We can only speculate why JHa-resistant lines were able to extend their life span without a concomitant reduction in fecundity. Two general mechanisms might account for the antagonistic physiological effects of JH on reproduction and survival (Tatar and Carey 1995; Barnes and Partridge 2003). On the one hand, JH might direct the allocation of energy (nutrients) toward reproduction, thereby withdrawing limited resources from investment into somatic maintenance and repair. On the other hand, JH might promote reproductive processes that directly accelerate damage-inducing processes independent of resource allocation. Although we cannot presently distinguish between these alternatives, recent evidence suggests that JH promotes reproduction, but is a negative regulator of stress resistance and immune function (Salmon et al. 2001; Tatar et al. 2001b; Rolff and Siva-Jothy 2002; Rantala et al. 2003; Flatt et al. 2005; Tu et al. 2006).

Fecundity is, however, not always negatively correlated with longevity. A heterozygous mutant genotype of *chico* (*chico*^{1}/*chico*$^{+}$) is JH-deficient and long-lived, but has a normal number of ovarioles (Clancy et al. 2001; Tu et al. 2005). Similarly, adult wild-type flies that were yeast deprived as third instar larvae exhibit reduced JH synthesis at eclosion, decreased ovariole number and fecundity, but show normal rates of aging (Tu and Tatar 2003). Thus, there is growing evidence that reproduction and survival can be to some degree uncoupled, and that the trade-off between these two traits is highly context dependent (Barnes and Partridge 2003; Partridge et al. 2005). Because many trade-offs are condition dependent (e.g., Stearns 1989, 1992, and references therein), it is possible that, under benign laboratory conditions (i.e., high nutrition), a slight increase in investment into somatic maintenance and survival would not require diverting resources from reproduction. Indeed, the long-lived *Drosophila* mutant *Indy* only exhibits reduced fecundity on a reduced-calorie diet, but not under normal (high calorie) rearing conditions (Marden et al. 2003). Similarly, certain mutants of *C. elegans age-1* and *daf-2* are long-lived without paying a fitness cost under normal laboratory conditions, but fitness costs of longevity become apparent when these mutants are exposed to nutritional stress or competed against a wild-type strain (Walker et al. 2000; Jenkins et al. 2004).

The effects of dietary application of JHa seen in our experiment were likely due to its JH activity. JHa can specifically mimic JH action in *Drosophila* (both in flies and cell culture) and other insects (Cherbas et al. 1989; Riddiford and Ashburner 1991; Wilson 2004). Importantly, effects of dietary JHa application typically recapitulate those of topical application (Wilson and Fabian 1986; Riddiford and Ashburner 1991; Wilson et al. 2003). In support of this, the natural compound JH III reduced the viability of our unselected JHa-susceptible flies, but JHa-resistant selected flies were insensitive to this effect (data not shown; Flatt 2004b). Furthermore, we found that the JHa concentration used in our experiment and assays increased fecundity of control flies. Thus, this dosage had a physiological effect consistent with the well-known role of JH in regulating vitellogenesis, ovarian maturation, and fecundity (Nijhout 1994; Hoffmann 1995; Gäde et al. 1997; Flatt et al. 2005). Although we cannot rule out that JHa selectively killed individuals with low fecundity, it is more parsimonious to assume that JHa treatment increased fecundity in control flies due its pro-reproductive, JH-like action.

Our results add to a growing number of studies showing that hormones are involved in mediating life-history trade-offs in a variety of organisms (Ketterson and Nolan 1992; Finch and Rose 1995; Dingle and Winchell 1997; Zera and Harshman 2001; Flatt et al. 2005; and references therein). In insects, selection experiments suggest that JH regulates the trade-off between flight capability and reproduction in crickets (Zera and Zhao 2004; Zera 2006), and quantitative genetic experiments with *Drosophila methoprene-tolerant* (*Met*) mutants link JH signaling with life-history pleiotropy (Flatt and Kawecki 2004). Moreover, application of JH or JHa in flies and beetles promotes reproductive processes at the expense of stress resistance or immune function (Salmon et al. 2001; Rolff and Siva-Jothy 2002; Rantala et al. 2003); it also mediates the trade-off between gonad development and eye-span (a secondary sexual trait) in stalk-eyed flies (Fry 2006).

Our findings have broad implications, beyond JH signaling in *Drosophila* and other insects. JH functions downstream of insulin/IGF-1 signaling, an evolutionarily conserved nutrient sensing pathway coordinating growth, reproduction, diapause, and aging in animals as diverse as *C. elegans*, *Drosophila*, and rodents (Tatar et al. 2003). Although *C. elegans* and rodents do not produce JH, similar hormones downstream of insulin/IGF-1 might regulate reproduction and longevity in these organisms. Recent

work has identified two lipophilic hormones that modulate the effects of the reproductive system on life span in *C. elegans* (Motola et al. 2006; Broué et al. 2007); in rodents, thyroid hormone might play a similar role (Tatar et al. 2003; Flatt et al. 2006). These findings strongly suggest that the endocrine regulation of trade-offs, such as between reproduction and life span, is evolutionarily conserved. However, trade-offs at the physiological level do not necessarily imply the existence of evolutionary trade-offs. For example, although some physiological trade-offs might be genetically variable and contribute to an evolutionary trade-off, others might be fixed and lineage specific (Stearns 1989, 1992). Interestingly, although our results suggest that JH is a proximate mechanism underlying the trade-off between reproduction and life span, we could not convincingly show that JH signaling actually mediates the evolutionary trade-off between these traits. Nonetheless, our results indicate that *Drosophila* populations harbor genetic variation that affects JH signaling or metabolism (cf. Flatt and Kawecki 2004; Flatt 2004a), and that this genetic variation may mediate the evolution of longer life span. The rapid progress made by molecular biologists in identifying candidate mechanisms affecting life-history traits enables evolutionary biologists to determine whether there is standing genetic variance for such mechanisms in natural populations and whether they are under selection.

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