Targeting environmental and genetic aspects affecting life history traits

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Chapter 1

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The effects of larval density on adult life-history traits in three species of *Drosophila*

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Abstract

There is evidence that longevity and starvation resistance are determined by a common genetic mechanism. Starvation resistance in *Drosophila* strongly correlates with both fat content and longevity, and is affected by density during rearing. In this study we examine how three species, *D. melanogaster, D. ananassae* and *D. willistoni*, respond to three larval density treatments. Starvation resistance after adult eclosion, and after 2 days of feeding, and longevity were examined in each sex. *D. willistoni* reacted differently to larval density than the other two species. This species showed an effect of density on longevity whilst *D. ananassae and D. melanogaster* showed no such effects. The results also indicate that starvation resistance is not solely determined by fat content. Resistance to starvation at two time points after eclosion differed among species. This may reflect differences in resource acquisition and allocation, and we discuss our findings in relation to how selection may operate in the different species.

Keywords

Drosophila, starvation resistance, longevity, fat, feeding, life history

Introduction

Longevity and starvation resistance are key life history traits and are studied in a wide range of organisms including, the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the mouse *Mus musculus* (Longo and Fabrizio 2002; Partridge and Gems 2002). Their importance to the mechanisms of ageing in part explains the interest in these traits. Several authors working with *Drosophila melanogaster* have found that longevity and starvation resistance are correlated (e.g. Zwaan et al. 1991; Chippindale et al. 1993). Others found that selection on starvation resistance can increase longevity (Rose et al. 1992; Harshman et al. 1999b) and *vice versa* (Zwaan et al. 1995a). This indicates not only that genes for longevity affect stress resistance, but that longevity is also affected by genes involved in stress resistance.

The genetics of longevity are beginning to be unravelled and current insights reveal an important role for hormones (*e.g.* the insulin pathway, Partridge and Gems 2002; ecdysone, Tatar et al. 2003, both in *Drosophila melanogaster*). Superimposed on these mechanisms are the environmental factors that affect life span and ageing (Tu and Tatar 2003; Zwaan 2003), including larval density (Miller and Thomas 1958). In this study, we focus on the interaction between longevity and starvation resistance in relation to larval rearing conditions for three species of *Drosophila*.

Longevity, starvation resistance and fat-content all show positive responses to higher larval density (Miller and Thomas 1958; Lints and Lints 1969; Luckinbill and Clare 1986; Zwaan et al. 1991; Robinson et al. 2000; Sorensen and Loeschcke 2001). Borash and Ho (2001) confirmed that in lines of *D. melanogaster* selected for survival at high larval density, resistance to starvation increased compared to unselected controls. In addition, Mueller et al. (1993) found that *D. melanogaster* lines reared at high densities showed higher starvation resistance than the same lines when reared at low densities. These results demonstrate that larval density is an important factor in shaping life histories, and that stocks generally show an increase in starvation resistance when reared at high densities.

Selection lines for higher longevity in *D. melanogaster* showed elevated lipid content later in life (Djawdan et al. 1996), and in general starvation resistance positively correlates with fat content (Zwaan et al. 1991; Graves et al. 1992; Djawdan et al. 1998). Relative fat content is a measure for the amount of energy available per unit of body mass. It follows that more energy reserves should result in a higher starvation resistance (Chippindale et al. 1996; Djawdan et al. 1998; Zera and Harshman 2001; Marron et al. 2003). Based on the links between longevity, starvation resistance and fat content, the last of these traits is thought to be an indicator of starvation resistance.

In a study of the ecological importance of food availability for *Drosophila* dispersal, Sevenster and Van Alphen (1993) examined whether the adult food uptake during the first two days after eclosion was utilised to increase starvation resistance. In contrast to expectation, the overall analysis for several species of *Drosophila* showed that starvation resistance was decreased after 2 days of food. This interaction between food availability and starvation resistance in adults of *Drosophila* species implies that food availability triggers processes such as resource allocation or the onset of a different metabolism that reduce starvation resistance. This finding might

relate to the trade-off between reproduction and longevity (Chippindale et al. 1993). However, starvation resistance appears to be variable among species of *Drosophila* (Sevenster and Van Alphen 1993).

Thus, starvation resistance and longevity are clearly related characters, and this relationship is modulated by larval density. In this study, we examine whether three closely related species of *Drosophila* show similar responses in adult starvation resistance and longevity to rearing at three different larval densities. We perform a detailed experimental analysis of the density effects on these life history traits for each species and each sex. Fat content is also measured in relation to starvation resistance. We examine whether the responses to larval density are the same for the different species, and for males and females. The responses are recorded for starvation resistance after hatching (SR), starvation resistance after two days of food (SR2), and longevity (L). In addition, we studied fat content directly after hatching, after two days of adult feeding and two days of starvation.

Materials and methods

Stock and maintenance

The *Drosophila* species, *D. ananassae*, *D. melanogaster* and *D. willistoni*, were collected in Panama in 1998 (Krijger et al. 2001). The size of the founding population exceeded 40. The flies were maintained at population sizes of approximately 200 individuals. All maintenance and experiments took place at 25ºC and 60% RH at a 12/12 D/L cycle. Stocks were originally cultured in bottles containing 80 ml vermiculite, 40 ml water and approximately 25 gr. banana soaked in yeast suspension and enriched with 4 ml propionic acid per liter water. A foam stopper containing a small drop of honey served as additional food for the adult flies and sealed off the bottles. In 2002, the stocks were switched to bottles containing 24 ml standard medium (20 gr. agar, 9 gr. kalmus, 10 ml. nipagin, 50 gr. saccharose and 35 gr. granulated yeast per liter water). Population sizes were subsequently maintained at 800 for each species. Pieces of paper towel were added to these bottles when flies were in their third instar larval phase to allow successful pupation. Harvesting and transfer to fresh bottles was done by aspiration and shaking. Prior to the current experiment, the flies were maintained in these new conditions for 10 generations. Flies were anaesthetised briefly with CO_2 for sexing and transfer to new vials for experimental purposes.

Experimental set-up

Traits

Starvation resistance (SR) is the time between eclosion of the adult from the pupa and death of the adult under conditions without food. This was measured on 6 ml agar medium (20 gr. agar 9 gr. kalmus, 5 ml. propionic acid, 5 ml. nipagin per litre

water) to prevent the flies from dehydrating and dying of desiccation. SR2 is the time between the onset of starvation for the adult (2 days after hatching from the pupa) and death. SR2 flies were first kept on standard medium for two days after which they were transferred to agar vials. Longevity (L) is the time between eclosion of the adult from the pupa and death of the adult under standard food conditions (see above).

Larval density

Groups of approximately 100 flies were allowed to lay eggs for 24 hours on small plates containing agar medium and wet yeast. Eggs were counted the following day and groups of 10-20 (low), 50-70 (medium) and 150-170 (high) eggs were put into vials containing standard medium. These densities match values from the literature (*e.g* Graves and Mueller 1993). However, food conditions as used in the laboratory vary widely. Thus, Perez and Garcia (2002) used a more nutritional medium than Zwaan et al. (1991); the latter study used a similar medium to that used in this study. Chapman and Partridge (1996) found that the food conditions for optimal longevity in *D. melanogaster* are close to the medium used in this study. Krijger's data (2000) indicate that these species have similar development times. However, it should be noted that other features, which we do not consider here, may be differentially affected for the three species.

For all traits, observed flies were maintained in groups of 10 individuals per vial containing 6 ml standard medium, with males and females separated. Treatment effects were tested by examining 5 replicate vials containing 10 flies, for each sex and each of the 3 species, subjected to 3 densities. This resulted in a grand total of 2700 flies. Vials for both of the starvation resistance treatments were changed every two days to minimise bacterial influences due to rapid bacterial growth (Borash and Ho 2001). For each experiment dead flies were removed daily from each vial to minimise disease and feeding from the corpses. In all assays flies were checked for alive or dead status by physical stimulation. Vials for longevity were changed each week to prevent flies from sticking to the medium.

Fat content

Fat content data were obtained in a separate experiment and was measured in flies harvested within 8 hours of eclosion for the determination of relative fat content. For measuring the fat content after two days of food or starvation, flies were kept on standard medium or agar medium, respectively, and were isolated after 48 hours of treatment. Flies were kept in vials with sexes separated to measure virgin fat content. Only live flies were analysed for fat content. Three replicates of 50 individuals each were checked per treatment. For the measurement of fat content over time we examined flies each day until all flies had starved to death. Five individuals of each line and each replicate were isolated and stored at –80ºC until further analysis. Flies were dried at 60ºC for 24h and then weighed on a Sartorius® ultra microbalance to determine dry weight. Fat was extracted by adding 1 ml of diethyl ether under continuous shaking (200 rpm) for 24 hours. The flies were dried for 24h at 60ºC and then re-weighed. The fat-free dry weight value was subtracted from the dry weight value. *D. melanogaster* flies were measured in groups of 5, while *D. ananassae* and *D. willistoni* flies were measured as individuals. Relative fat content was calculated by dividing absolute fat content by dry weight (Zwaan et al. 1991).

Comparison between starvation resistance after eclosion and after two days of feeding

To make a clear comparison between the SR directly after eclosion and that after two days of feeding, we subtracted the first two days of feeding from SR2. Thus, we compared the actual time of death after the onset of starvation.

Statistics

Figure 1 illustrates the outline of the experimental set-up for the SR, SR2 and L-measurements. All data were initially analysed to detect general patterns, but specific effects of density on the three life history traits were tested in separate analyses for each species and sex. We used Tukey HSD tests to examine whether the effect of density differed between groups or within treatments (Figure 2). In addition, differences between the sexes of each species for starvation after two days of food were compared to those of starvation after eclosion.

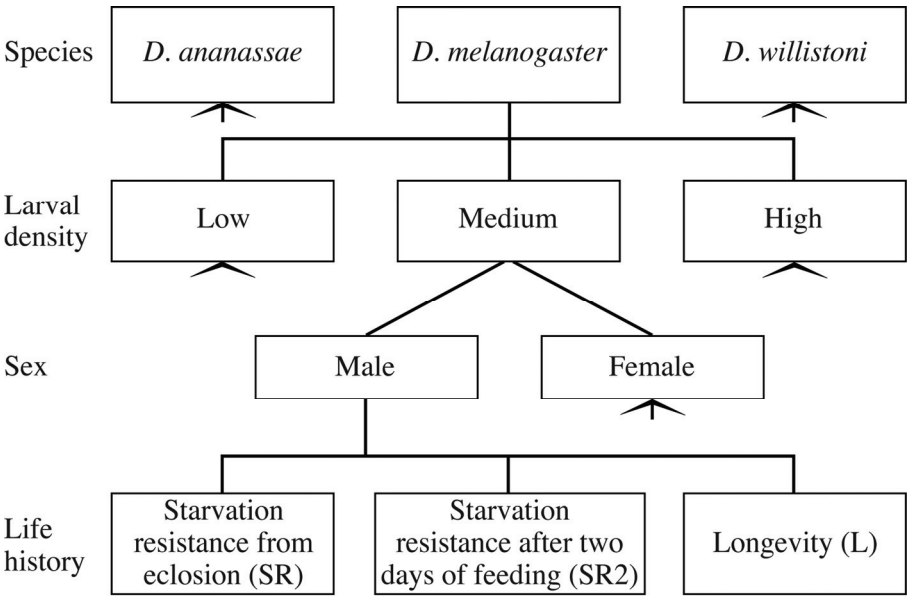

Figure 1. Set up of the experiment. The statistical analyses were applied at each horizontal level.

The data for longevity and for starvation resistance after hatching and after 2 days of food were not normally distributed (Shapiro-Wilkinson W test, data not shown). No transformation (lognormal, Weibull and Weibull with threshold) resulted in normalisation. However, tests with non-parametric models could not examine the interaction factors that are the main concern of our paper. We, therefore, determined whether the deviations from normality could bias our conclusions. There was no general pattern, such that one species, treatment or sex mainly contributed to the non-homogeneity of the variances. This suggested that using parametric models (ANOVA) would not bias these deviations from normality. So we used such models and interpreted the results with caution.

Dry weight, fat content and relative fat content data were not normally distributed, yet variances were homogeneous. As reasoned above, we still tested for differences between samples using ANOVAs. All statistical tests were performed using JMP 5.0.1.

Results

Starvation resistance

The overall analysis shows a significant negative effect of increasing density on starvation resistance. The analysis of SR showed significant effects of species ($F_{2,852}$=494.36, P<0.0001), sex ($F_{1,852}$=42.31, P<0.0001), and density ($F_{2,852}$=29.22, P<0.0001); see Figure 2. A significant species*sex interaction ($F_{2,852}$=48.73, P<0.0001) indicates that the sexes behave differently among species. This is largely explained by the differences between males and females in *D. melanogaster*.

Species-specific analysis

SR was significantly higher in female than male *D. melanogaster* ($F_{1,295}$=86.65, P<0.0001), whereas such differences were absent in *D. ananassae* and *D. willistoni* ($F_{1,269}$=0.42, P=0.53 and $F_{1,288}$=1.02, P=0.33, respectively.) SR decreased significantly with increasing larval density in *D. melanogaster* ($F_{2,295}$= 8.13, P=0.0004), *D. willistoni* ($F_{2,288}$=3.53, P=0.03) and *D. ananassae* ($F_{2,269}$=27.20, P<0.0001).

Sex-specific analysis

SR showed no significant density effects in either sex of *D. melanogaster* (females: $F_{2,149}$=0.49, P=0.62; males: $F_{2,146}$=0.24, P=0.78). Sex-specific analysis of *D. ananassae* and *D. willistoni* was not performed because no differences were found between the sexes in the species-specific analyses.

Longevity

For longevity, both species and density were significant factors ($F_{2,794}$=139.52, P=<0.0001; $F_{2,794}$=12.4, P<0.0001, respectively). However sex differences were not

found for this trait ($F_{1,794}$=1.74, P=0.19). The species*sex*density interaction showed a significant effect ($F_{4,794}$=2.48, P=0.043)

Species-specific analysis

Density was not important for *D. melanogaster* and *D. ananassae* longevity ($F_{2,272}$=0.93, P=0.40 and $F_{2,265}$=1.47, P=0.23, respectively), but was for *D. willistoni* ($F_{2,257}$=14.06, P<0.0001). The latter species also showed a significant sex*density interaction ($F_{2,257}$=5.18, P=0.0062). This is clear from Figure 2, male longevity decreased monotonically with density, whilst female longevity was lowest for intermediate densities. This largely accounts for the significant species*sex*density interaction found in the overall analysis.

Starvation resistance after two days of food

SR2 showed similar results to the SR treatment for the factors, species and sex ($F_{2,854}$=49.27, P<0.0001 and $F_{1,854}$=77.1, P<0.0001). Density effects were also significant ($F_{2,854}$=6.67, P=0.0013), and there was a significant species*sex interaction factor ($F_{2,854}$=27.82, P<0.0001).

Species-specific analysis

Density affected this trait differently in the sexes and the species in a complex fashion, and therefore no clear pattern can be distilled from the data (see also figure 2). Sex effects on SR2 were significant for both *D. melanogaster* and *D. ananassae* ($F_{1,288}$=356.95, P<0.0001 and $F_{1,286}$=29.60, P<0.0001), but not for *D. willistoni* ($F_{1,280}$=0.45, P=0.50). Larval density was important for SR2 in *D. melanogaster* ($F_{2,288}$=31.65, P<0.0001) and *D. willistoni* ($F_{2,280}$=3.4371, P=0.034), but not for *D. ananassae* ($F_{2,286}$=2.94, P=0.09). In addition, *D. melanogaster* and *D. willistoni* showed significant sex*density interactions ($F_{2,288}$=31.14, P<0.0001 and $F_{2,280}$=8.23, P=0.0003). *D. willistoni* males showed their highest starvation resistance at medium density whereas females showed their lowest starvation resistance at that density. *D. melanogaster* showed a slight decrease in SR2 with density in both sexes.

Sex-specific analysis

D. melanogaster females ($F_{2,147}$=0.66, P=0.52) and males ($F_{2,141}$=2.66, P=0.073) showed no significant effect of density on SR2. This trait in *D. ananassae* was independent of density in males ($F_{2,141}$=0.44, P=0.64), but was density-dependent in females ($F_{2,145}$=3.76, P=0.026).

The indication that density is a factor in shaping this trait comes only from the overall analysis, and from the species-specific analysis where *D. melanogaster* and *D. willistoni* showed significant effects of larval density. Higher order interactions indicate differential responses of the sexes to density.

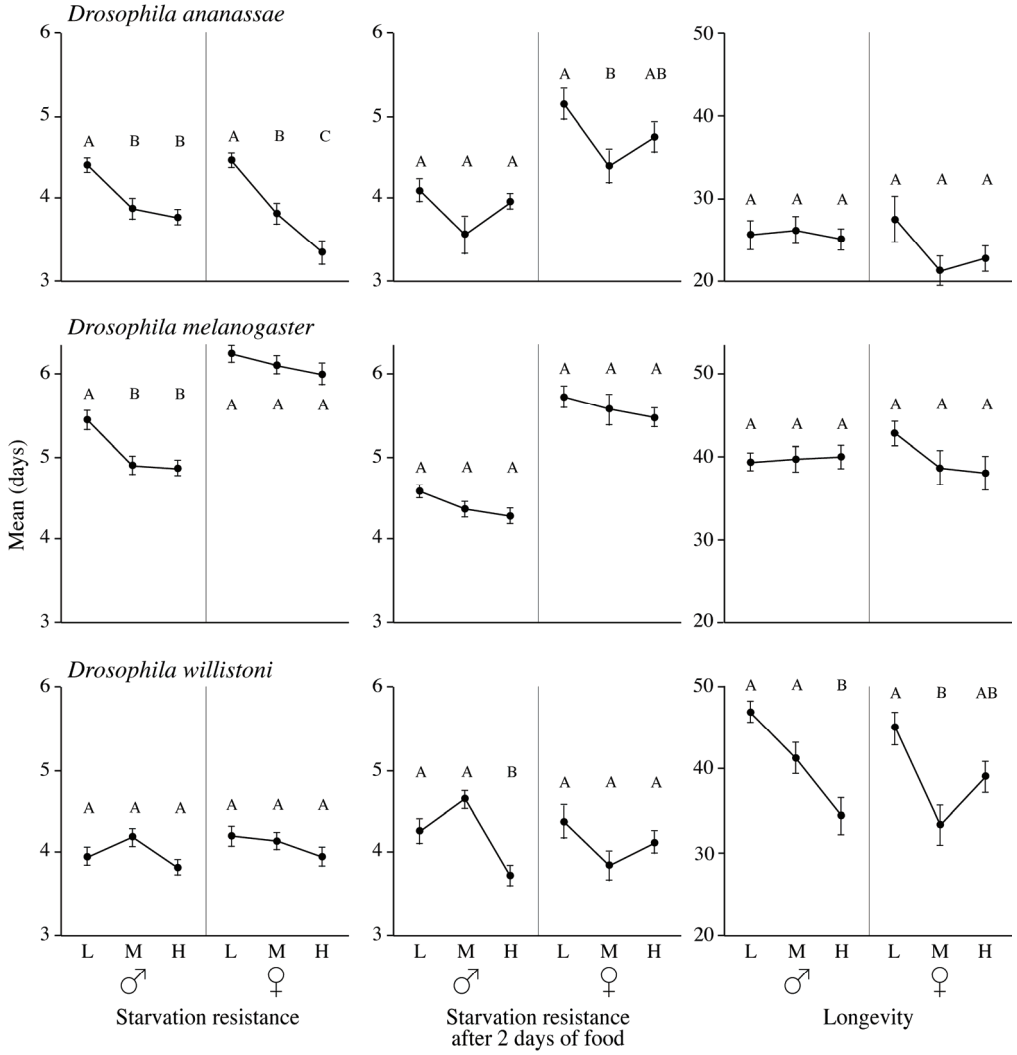

Figure 2. The average survival of males and females of three species of *Drosophila* across three larval density treatments (L, low density; M, medium density; H, high density). Life span is measured under conditions of starvation from eclosion, two days of feeding after eclosion and *ad libitum* feeding. Letters indicate significant differences between groups (after *post hoc* Tukey testing).

Dry weight and fat content

Dry weight

The size of *D. melanogaster* and *D. willistoni* females declined with increasing larval density (12% decline, $F_{2,6}$=6.19, P=0.035; 12 % decline, $F_{2,27}$=6.84, P=0.0039, respectively), whereas males showed no significant affect (5% decline, $F_{2,6}$=0.85, P=0.47; 3% decline, $F_{2,27}$=0.34, P=0.72, respectively). The density effects were not significant in *D. ananassae* (females 13% decline, $F_{2,27}$=3.17, P=0.058; males 2% decline, $F_{2,27}$=2.15, P=0.1358), though the responses for the sexes were similar to those of the other species and the decline in female size approached significance. In general, females of each species showed a stronger reduction in dry weight with increasing density than males. This effect on body size suggests that the animals were mildly stressed by higher larval density.

Fat-free dry weight

Females of all three species showed a negative effect of density on fat-free dry weight (*D. melanogaster* 15% decline, $F_{2,6}$=10.27, P=0.016; *D. willistoni* 9% decline, $F_{2,27}$=4.46, P=0.021; *D. ananassae* 15%, $F_{2,27}$=3.75, P=0.037), whilst this only occurred for males in *D. melanogaster* (15% decline, $F_{2,6}$=28.18, P=0.0015). Males of *D. ananassae* and *D. willistoni* showed non-significant declines in fat-free dry weight (6% and 5% decline, respectively) .Figure 3 illustrates the sensitivity of females with a general decline in fat-free dry weight with higher larval density.

Fat content

D. melanogaster and *D. ananassae* males showed effects of increasing larval density on adult fat content ($F_{2,6}$=5.30, P=0.047; $F_{2,27}$=5.54, P=0.0097, respectively), whereas females did not ($F_{2,6}$=2.94, P=0.13; $F_{2,27}$=0.09, P=0.91, respectively). *D. willistoni* showed a significant density effect in female fat content ($F_{2,27}$=7.32, P=0.0029) but not in males ($F_{2,27}$=1.46, P=0.25).

Relative fat content

Relative fat content in *D. melanogaster* and *D. ananassae* showed a strong effect of larval density ($F_{2,15}$=7.54, P=0.005; $F_{2,57}$=7.01, P=0.0019, respectively). Of these species males showed significant responses ($F_{2,6}$=6.33, P=0.005 and $F_{2,6}$=12.01, P=0.008, respectively) whereas females showed none ($F_{2,27}$=4.76, P=0.058 and $F_{2,27}$=1.85, P=0.18, respectively). In contrast, there was no effect in *D. willistoni* ($F_{2,56}$=1.49, P=0.23). *D. willistoni* males showed no significant effect ($F_{2,27}$=1.92, P=0.17) whilst females showed an effect ($F_{2,27}$=5.48, P=0.01). Although not all tests showed significant effects of density, the generally positive trend with increasing density is consistent with the findings of Zwaan et al. (1991).

The effect of 2 days of starvation on relative fat content

Each species showed significantly reduced relative fat content after two days of starvation as compared to immediately after adult eclosion (Table 1).

Table 1. Percentage decline of relative fat content during starvation. F- and P-values of the ANOVA test show whether relative fat content was affected when animals were first kept for 2 days without food as compared to measurement directly following eclosion. All species showed a decline in fat content. MLG, *D. melanogaster*; WLS, *D. willistoni*; ANS, *D. ananassae*.

	MLG	WLS	ANS
Overall	$F_{1,6}$=153.11; P<0.0001	$F_{1,8}$=7.88; P=0.0229	$F_{1,7}$=13.04; P=0.0086
♂	74% $F_{1,3}$=152.02; P=0.0011	16% $F_{1,4}$=0.46; P=0.5351	39% $F_{1,4}$=9.85; P=0.0349
♀	57% $F_{1,3}$=27.82; P=0.0133	60% $F_{1,4}$=13.47; P=0.0214	58% $F_{1,3}$=5.95; P=0.0926

Comparison of starvation resistance after 0 and 2 days of feeding.

The overall analysis of these two traits showed significant species, sex and larval density effects ($F_{2,1704}$=306.28; P<0.0001, $F_{1,1704}$=124.26, P<0.0001 and $F_{2,1704}$=28.10, P<0.0001, respectively). The factor treatment was not significant ($F_{1,1704}$=0.44, P=0.5081, but see Table 2) indicating that there is no overall difference between SR and SR2. All interaction factors, including the species*sex*density interaction ($F_{4,1704}$=4.62, P=0.001), were significant except for those involving the factor treatment.

Table 2. P-values of the ANOVA test of starvation resistance from eclosion (SR0) versus starvation resistance after two days of feeding (SR2) specified per species, sex and larval density; MLG, *D. melanogaster*; WLS, *D. willistoni*; ANS, *D. ananassae*; (+) means that SR0>SR2; (-) means that SR0<SR2.

	MLG ♂	MLG ♀	WLS ♂	WLS ♀	ANS ♂	ANS ♀
Low	<0.0001, +	0.0045, +	0.1143, -	0.4494, -	0.1082, +	0.0023, -
Medium	0.0002, +	0.0187, +	0.0040, -	0.1309, +	0.2762, +	0.0159, -
High	<0.0001, +	0.0021, +	0.5073, +	0.2420, -	0.1852, -	<0.0001, -

D. melanogaster males and females showed similar results for the SR-SR2 comparison to those found by Sevenster and Van Alphen (1993) at all densities. *D. melanogaster* flies generally resisted starvation better directly after hatching than after two days of food (Table 2). SR2 of *D. ananassae* males was not affected compared to SR at each density, while female SR2 was significantly higher at all densities. *D. willistoni* males were significantly different only at medium density whereas they were not at the other densities or in females (Table 2). This implies that

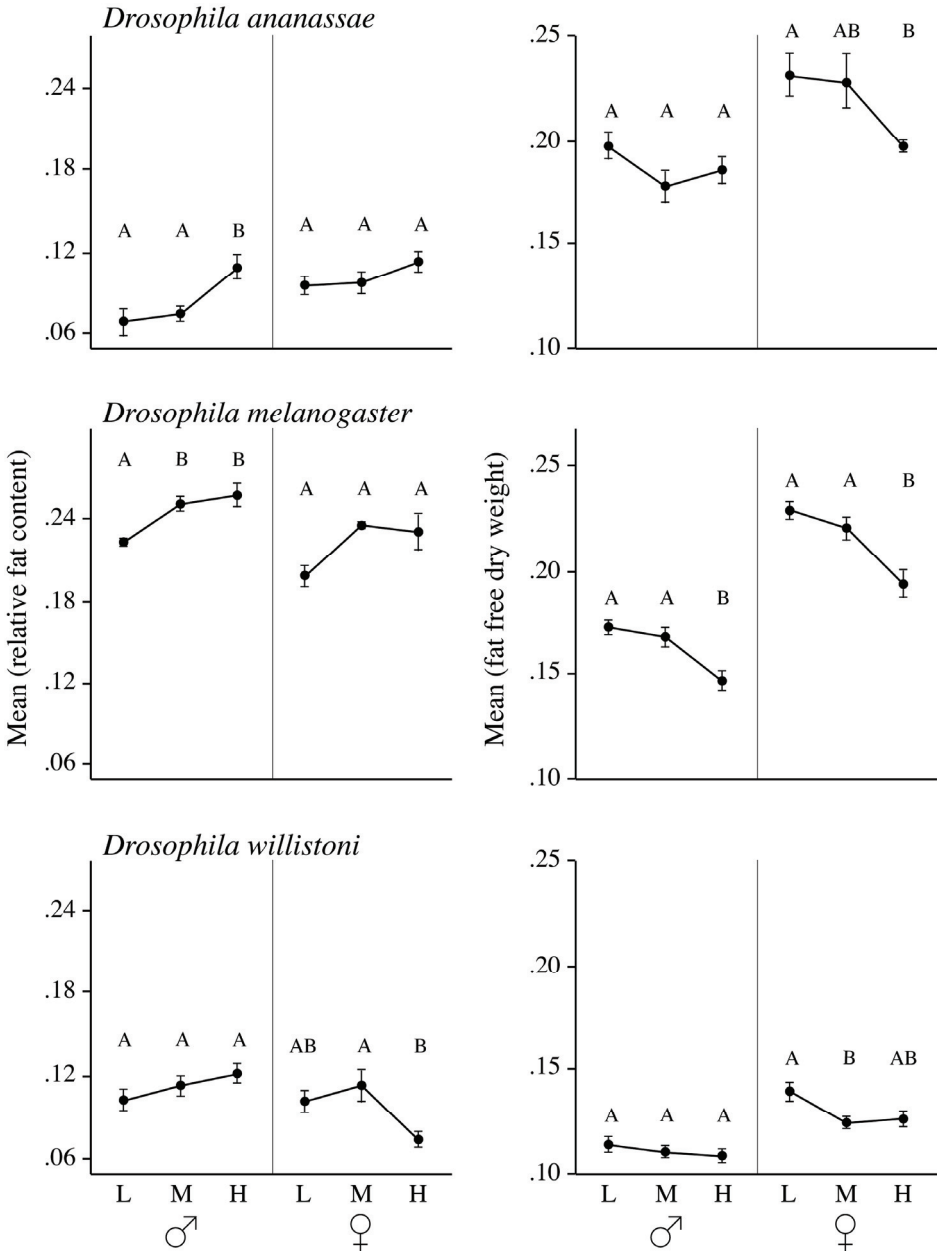

Figure 3. The average relative fat content and fat-free dry weight of males and females of three species of *Drosophila* across three larval density treatments (L, low density; M, medium density; H, high density).

for *D. willistoni*, food availability in the first two days after eclosion does not affect starvation resistance.

In general, *D. melanogaster* shows a significant decrease in starvation resistance after feeding in the adult stage. *D. willistoni* did not show differences between SR and SR2, whereas *D. ananassae* showed a significant increase in female starvation resistance. We conclude that larval density affects species and sexes in different ways thus accounting for the significant species*sex*density interaction.

Discussion

We examined effects of larval density on adult life history traits and physiology in three species of *Drosophila*. In general, the responses were similar among species, however a more detailed comparison revealed species and sex-specific responses. We will discuss whether the responses reflect phylogenetic relationships. Each of the species showed a different response of starvation resistance after adult feeding. The effect of post-eclosion feeding and the use of standard medium will also be discussed. Larval density affected dry weight and fat content in a similar way among the species. We will describe in more detail how larval density influences life history traits, and we will elaborate on the effects larval density has on fat and starvation resistance, and whether there is a causal link between these two traits. Finally we will speculate on the underlying genetics of the traits studied.

Effects of rearing conditions match phylogenetic relationships of species

D. ananassae, D. melanogaster and *D. willistoni* showed significant effects of larval density on adult starvation resistance directly after eclosion and after two days of food. We did not find effects of larval density on longevity in *D. ananassae* and *D. melanogaster*, whereas we did in *D. willistoni*. Apparently crowded larval conditions in *D. willistoni* decrease adult longevity, contrasting with the increase in longevity found in *D. melanogaster* studies (Miller and Thomas 1958; Zwaan et al. 1991). *D. melanogaster* and *D. ananassae* are phylogenetically more closely related to each other than either is to *D. willistoni* (O'Grady and Kidwell 2002). Although we used only three observations for each sex of each species, the responses of underlying physiology to larval densities seem to reflect these phylogenetic associations. Further research into how life histories reflect phylogenetic associations would be extremely valuable.

The effects of post-eclosion feeding on starvation resistance

Two days of feeding after adult eclosion in *D. melanogaster* led to a subsequent decrease in starvation survival relative to flies starved directly from eclosion. Apparently the opportunity to feed after eclosion was not used to increase energy reserves but might have triggered different metabolic processes in *D. melanogaster*, such as reproduction. Such mechanisms are known from yeast (Lin et al. 2002). Additionally, some authors have found that adding yeast to the diet of *D. melanogaster* initiated reproduction and diminished both SR and L (Chippindale et al.

1993; Simmons and Bradley 1997). This led Chippindale et al. (1993) to the finding that starvation resistance and longevity trade off against reproduction. Forced adult migration due to lack of food and oviposition site may trigger postponed reproduction and thereby influence the trade-off between longevity and reproduction. In contrast, finding food after adult eclosion may induce a reproductive response and in turn shorten starvation resistance and longevity. An eco-physiological explanation for the difference between SR and SR2 in *D. melanogaster* may be that females have a fitness advantage for early reproduction in a growing population and are capable of producing eggs within about 8 hours of eclosion. Therefore, *D. melanogaster* is likely to allocate resources to reproduction rather than to somatic maintenance under laboratory circumstances.

This scenario for *D. melanogaster* does not, however, fit our results for *D. willistoni* and *D. ananassae*. *D. ananassae* females showed an increase in SR after feeding, indicating that the food uptake prolonged, rather than shortened, SR. This is accompanied by a strong increase in relative fat content after two days of feeding in virgin *D. ananassae* (Table 3). In contrast, *D. willistoni* showed no difference in relative fat content after two days of food compared to directly after eclosion (Table 3). *D. willistoni* also showed no effect of larval density and adult feeding on starvation resistance, suggesting that there is little plasticity for starvation resistance in this species.

Table 3. F- and P-values of ANOVA on adult relative fat content at eclosion (RFC0) compared to relative fat content after 2 days of feeding (RFC2). MLG, *D. melanogaster*; WLS, *D. willistoni*; ANS, *D. ananassae*; (+) means that RFC0> RFC2; (-) means that RFC0< RFC2.

	MLG	WLS	ANS
overall	$F_{1,7}$=0.96; P=0.36, +	$F_{1,8}$=0.21; P=0.66, +	$F_{1,8}$=14.96; P=0.0048, -
♂	$F_{1,3}$=0.11; P=0.76, +	$F_{1,4}$=0.07; P=0.80, +	$F_{1,4}$=27.24; P=0.0064, -
♀	$F_{1,3}$=3.45; P=0.14, +	$F_{1,4}$=0.29; P=0.62, +	$F_{1,4}$=1.57; P=0.28, -

Feeding media

Different species of *Drosophila* could respond differently to the same larval medium, and this could introduce an artefact into our comparisons. The standard medium is widely used for *D. melanogaster* and may be less suitable for development of the other species. Non-optimal developmental conditions might then induce less optimal adult phenotypes. More natural food conditions, such as banana, are more likely to vary in nutrient concentrations and in resources thus introducing environmental variation. In *D. melanogaster* food supplements can increase longevity (Takahashi et al. 2001) and starvation resistance (Zinke et al. 2002). We reared all species on standard medium for several generations before our experiment to reduce such effects whilst retaining the ability to make direct comparisons.

Fat content and dry weight

Female dry weight and fat-free dry weight declined in response to larval density. Males had a more uniform dry weight over different larval densities. Generally, fat

content increased for all species and sexes with increasing larval density, whilst dry weight and fat-free dry weight decreased with increasing density in most species. This indicates that the higher larval densities should be regarded as a stress, as is consistent with other studies (*e.g.* Zwaan et al., 1991). Djawdan et al. (1998) showed that glycogen and lipid energy content when pooled give the best indicator of the ability to resist starvation in *D. melanogaster*. However, Graves et al. (1992) clearly showed that flies with no glycogen reserves are equally capable of resisting starvation as glycogen-containing control flies. Experiments with *D. melanogaster* lines also indicated that glycogen content was not affected by selection for starvation resistance whereas fat was (Baldal et al. unpublished results). Thus, fat appears to be the most important energy reserve underlying starvation resistance (Zwaan et al. 1991; Djawdan et al. 1998).

Density effects

Zwaan et al. (1991) found that high-density flies have the highest fat content, facilitating an increased starvation resistance. The present study does not show this; animals from high larval densities showed the shortest starvation resistance. Borash and Ho (2001) found that even though flies selected at higher larval density showed an increase in starvation resistance compared to controls when reared at normal larval density, a reduction in starvation resistance was observed when larval density increased. One explanation for these seemingly conflicting results is that Zwaan et al. (1991) measured starvation resistance 21 days after eclosion whereas in both Borash and Ho's and our own study, starvation resistance was measured directly after hatching. We found that exposure to higher larval density reduces body size. This is in line with Santos et al. (1994), who found a decreased fitness and thorax length, and thus body size, at increasing densities similar to those observed in the present study. However, compared to Zwaan et al. (1991), the reduction in body size is small. The effects of larval density in the present study tend to be small but are likely to reflect important responses to developmental conditions. Thus, we conclude that higher larval densities lead to smaller flies with a high relative fat content and reduced starvation resistance.

Fat, starvation resistance and longevity

Starvation resistance is associated with longevity, suggesting that these traits share molecular pathways. Reproduction and starvation resistance are both dependent on fat reserves and, therefore, we hypothesise a trade off between them. In the present study, relative fat content increases, and starvation resistance decreases with increasing larval density and *vice versa*. Changing density causes many physiological changes, including relative fat content. Starvation resistance is in turn affected by fat storage, as well as by at least some of the other changes. If starvation resistance does not solely depend on the amount of fat, then additional regulatory mechanisms must also play a role. Leroi (2001) argued that the insulin-signalling pathway is very important in shaping the trade off between longevity and reproduction. If this is so, then resource allocation can not be solely responsible for the trade off between longevity and reproduction, and thus neither of the traits is

dependent only on resources. This fits our finding that starvation resistance can be influenced by factors other than stored fat reserves.

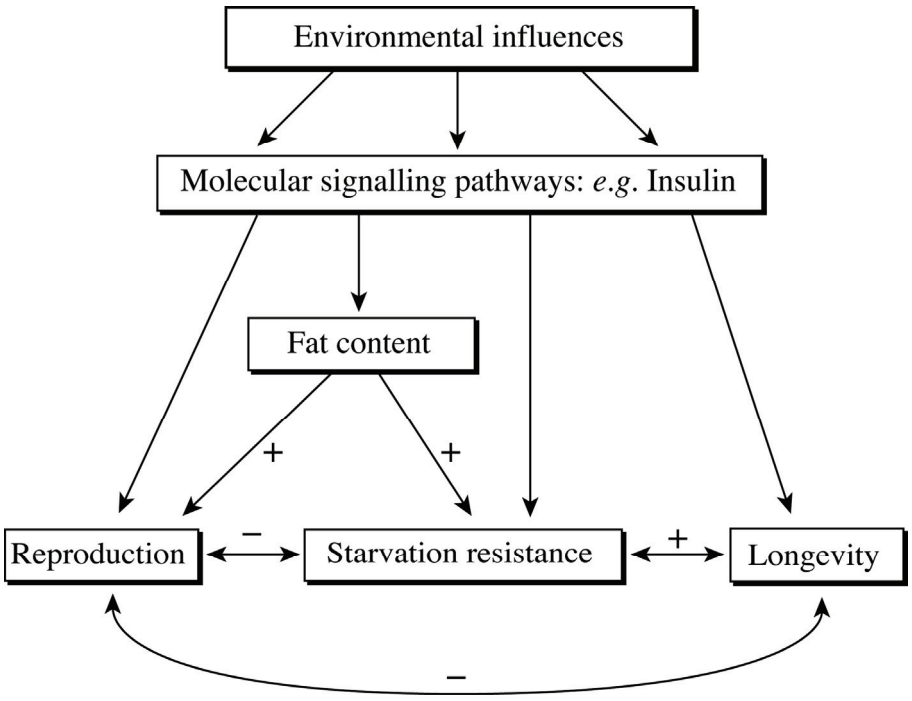

Figure 4. A model of how different factors may affect starvation resistance.A direct link is proposed between the molecular signalling pathways and starvation resistance. "+" Means that factors are positively correlated, "-" means that factors are negatively correlated.

Underlying genetic mechanisms

It is difficult to demonstrate that there is a particular molecular signalling component involved in increasing starvation resistance especially if such a pathway regulates both starvation resistance and the build up of fat. Thus fat acquisition could be an unavoidable by-product of an increase in starvation resistance and not necessarily causal for starvation resistance. Therefore, we propose that the resource allocation model for the trade off between longevity/starvation resistance and reproduction should attempt to incorporate such molecular signalling components (figure 4).

Modulating the genetic mechanism underlying any one of these life history traits would be expected to affect all the traits in a similar way if they share underlying physiological mechanisms. However, if additional pathways also affect the traits, modulation of one such pathway may not induce similar responses. Responses of SR

and longevity to larval density seem to follow a similar trend of reduced life span with increasing larval density in our experiments (Figure 2) suggesting that these life history characters share physiological mechanisms. Differences between the species lead us to believe that other regulatory mechanisms are also involved. Work of Force et al. (1995) implies the same; they found that some long-lived selection lines did not show a subsequent increase in starvation resistance. Two recent studies of *D. melanogaster* showed that initial correlations between functional traits can change when cultures are kept under continuous selection. Thus, Phelan et al. (2003) found that selection lines for longevity lost their stress resistance, whilst Archer et al. (2003) showed that selection lines for starvation resistance lost elevated longevity found earlier on after several generations. Therefore, caution is necessary before assuming that longevity and starvation resistance depend on the same genetic mechanism.

Conclusion

Different species of *Drosophila* show similar directions of response to density stress; it is the intensity of the response that differs per species. The sexes can also show different responses in different situations, probably because of differing reproductive demands across environments. High larval density acts as a stress environment and tends to lead to different resource allocation in adults with a shift from dry weight to fat content. However, a higher fat content does not lead to a higher starvation resistance or longevity. Access to food in adults also does not necessarily lead to higher starvation resistance, even though it induces higher fat content. These patterns differ in detail across species; the broad pattern is similar among species, but the fine-tuning and interdependence of the traits is not. How this is reflected on the genetic level, for example in the involvement of genetic pathways is the subject of our future research.