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Adult crowding effects on longevity in *Drosophila melanogaster*: Increase in age-independent mortality

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The manipulation of longevity through environmental treatments has provided many insights into the physiological processes affecting life-span. Here, we report the reduction of longevity in adult *Drosophila melanogaster* after three days of moderate adult crowding. Crowding is shown to reduce life-span through increased age-independent mortality rather than through altering the rate of ageing. Preliminary evidence also suggests that increased age-independent mortality after crowding may result from a reduction in stored energy reserves. The results also suggest that populations of *D. melanogaster* routinely maintained at high-adult densities may be relatively less susceptible to the detrimental effects of crowding on longevity.

THE study of ageing has long been approached at two different levels of biological organization: at the individual and sub-individual level by gerontologists, and at the populational level by demographers. In the past one-and-a-half decades, evolutionary biologists have tried to unite these divergent approaches by trying to understand how and why ageing evolves, an approach that combines demography, population genetics and physiology[1–3]. From this evolutionary viewpoint on ageing, longevity (or life-span) is seen as but one among a multitude of life-history traits, all interrelated as a consequence of shared underlying physiological constraints[2–10].

Genetic and environmental manipulation of longevity

Many studies on ageing in the past several years have focused upon genetic and environmental manipulations of life-span[2,3]. For example, increased life-span has successfully been selected for in *Drosophila melanogaster* by several workers independently[1,11–13]. Similarly, environmental manipulations such as dietary restriction[6], restricted mating[14–16], and the addition of urea to the food medium[17] have all been observed to enhance longevity in *D. melanogaster*. Similar effects of many environmental treatments on longevity have also been observed in a variety of other invertebrate[7,18–21] and vertebrate[22–24] species. Moreover, investigation of the physiological mechanisms underlying observed genetic and environmental effects on longevity has provided useful insights into the nature of the constraints placed by correlations among life-history characters upon the attainment of increased life-span under a given set of conditions[2,5,25–27].

Estimating the rate of ageing

The demographic perspective on ageing suggests another level at which the nature of the effect of various factors on life-span can be assessed, by focusing on rates of ageing, rather than on changes in life-history traits correlated with longevity. In recent years, several studies have focused on 'rates of ageing' as reflected by the α parameter of the Gompertz equation[28] that models age-specific mortality as an exponentially increasing function of age[17,24,29–32]. According to the Gompertz equation, the mortality rate at age x, $\mu(x)$, is given by

$$\mu(x) = Ae^{\alpha x},$$

where the two parameters, A and α, represent the age-independent mortality rate and the rate of increase in mortality rate with age, respectively: this latter parameter, α, is considered to represent the rate of ageing[2,18,29–32]. Results of some studies on *Drosophila*[17,32] strongly suggest that environmental factors affecting longevity tend to do so through their effect on the age-independent Gompertz parameter (A), whereas genetically induced changes in longevity tend to involve changes in the Gompertz 'rate of ageing' (α). There is also some evidence that, in the bruchid beetle *Callosobruchus maculatus*, mating *per se* may affect the Gompertz 'rate of ageing', whereas egg-production affects the age-independent Gompertz parameter[18].

Adult density and longevity

Adult density is an environmental factor that is known

to adversely impact a variety of fitness correlates in diverse species[33–39]. In *Drosophila*, adult crowding over a period of many days has been shown to significantly reduce longevity[33,37]. In the present study, we used a set of 15 populations of *D. melanogaster*, that had been subjected to life-stage-specific density-dependent natural selection in the laboratory, to seek answers to the following questions: (i) Can brief episodes of moderate levels of adult crowding significantly reduce longevity? (ii) Is the effect, if any, of adult crowding on longevity mediated through changes in the age-independent or age-dependent parameters of the Gompertz equation, or both? (iii) Are populations regularly maintained at high adult densities better able to withstand the deleterious effects, if any, of adult crowding on longevity?

Methodology

Experimental populations

This study used three sets of five replicate populations of *D. melanogaster* that had each been subjected to differing levels of larval or adult density for over 50 generations[40]. All populations were maintained on banana-molasses food at 25°C, continuous light, and had a generation time of about 3 weeks. Population sizes of each generation were of the order of 2000–4000 breeding adults. The five populations crowded as larvae (CU_1. . .CU_5) were reared at densities of 1000 or more larvae per 6 dram vial (2.2 cm d × 8.4 cm h). Eclosing adults were collected daily from these vials, and kept at a low density of about 60–80 adults per 8 dram vial (2.4 cm d × 9.5 cm h). The five uncrowded populations (UU_1. . .UU_5) were reared at low larval densities of 60–80 larvae per 8 dram vial; eclosing adults were subjected to the same density as the CU populations. The five populations crowded as adults (UC_1. . .UC_5) were reared at low larval densities of 60–80 larvae per 8 dram vial; eclosed adults were collected from these vials on the 13th day after egg-laying and kept in 8 dram vials at densities of about 160–200 adults per vial. The three sets of populations, thus, differed in the degree of larval or adult crowding to which they were exposed, with the UU populations acting as controls to both the UC and CU populations. Prior to initiating a new generation, all the eclosed adults from a population were dumped into a plexiglass cage (25.5 × 20 × 14.4 cm^3) and supplied with liberal amounts of live yeast paste for 2 days before egg collection. All three sets of populations were derived from the five B populations of Rose[1], each B population being used as the progenitor of one CU, one UC, and one UU population. Consequently, CU, UC and UU populations bearing the same numerical subscript are more closely related to each other, as compared to other populations subjected to the same density regime.

Collection of adult flies for longevity assay

Prior to initiating the longevity assay described below, all test populations were passed through one complete generation of identical rearing conditions, to eliminate any differences among selected lines due to environ-

Table 1. ANOVA for longevity of UU, UC and CU flies after three days of crowded (150 flies/vial) or uncrowded (8 flies/vial) conditioning (Cond). Longevity was assayed on flies kept in groups of 4 males and 4 females per vial. Significant fixed main effects and interactions are indicated in bold type (Blk, block, Sel, selection, Cond, conditioning treatment; parentheses are used to denote nested effects)

Source	*df*	MS	*F*	*P*
Block	4	1671.44	13.54	<0.0005
Selection	2	1246.85	1.59	>0.25
Conditioning	**1**	**4971.88**	**8.09**	**<0.05**
Sex	**1**	**39991.16**	**213.83**	**<0.0005**
Vial (Blk × Sel × Cond)	270	123.39	1.06	>0.20
Blk × Sel	8	782.73	6.36	<0.0005
Blk × Cond	4	614.69	4.99	<0.001
Blk × Sex	4	187.02	1.06	>0.25
Sel × Cond	2	350.29	1.69	>0.10
Sel × Sex	2	1038.40	3.35	>0.05
Cond × Sex	1	716.42	1.97	>0.10
Sex × Vial (Blk × Sel × Cond)	258	176.84	1.53	<0.0005
Blk × Sel × Cond	8	207.83	1.68	>0.05
Blk × Sel × Sex	8	310.70	1.76	<0.05
Blk × Cond × Sex	4	363.87	2.08	>0.05
Sel × Cond × Sex	2	152.79	0.50	>0.25
Blk × Sel × Cond × Sex	8	302.60	1.72	>0.05
Error	1525	115.92		

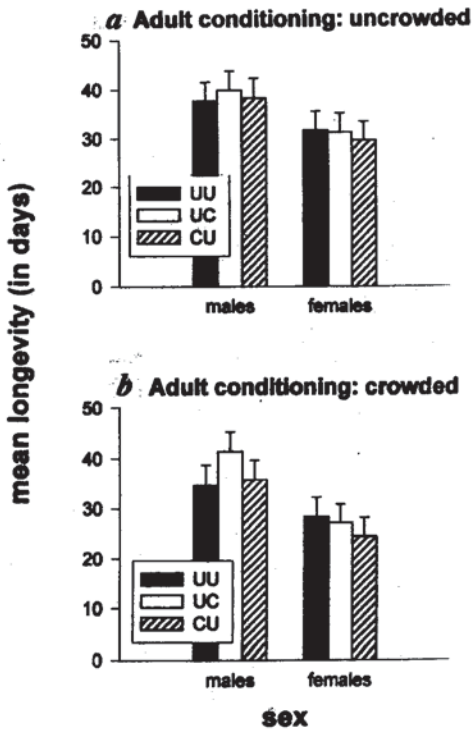

Figure 1. Mean longevity of flies after three days of uncrowded (*a*) or crowded (*b*) conditioning as adults. The error bars depict 95% confidence intervals about the mean of the five replicate populations of each selection regime, and were calculated using least squares estimates of the standard errors of cell means in the randomized block ANOVA.

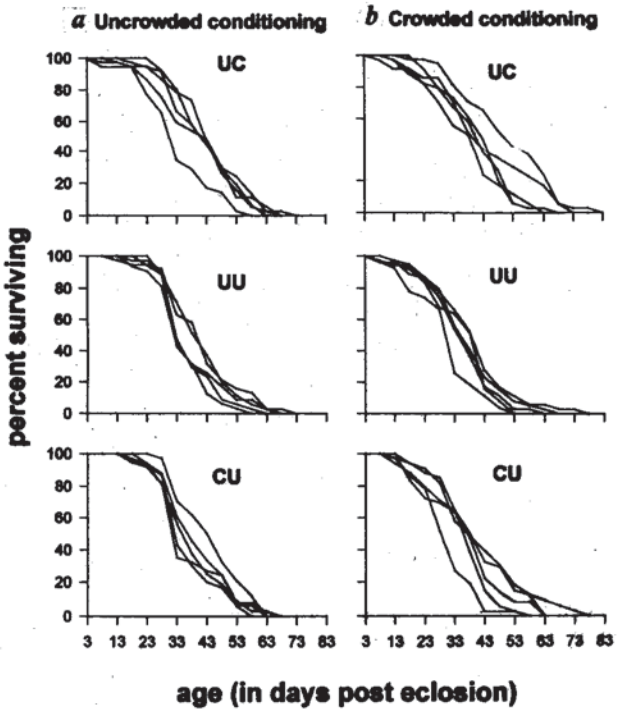

Figure 2. Per cent survival to various ages of adult males from each of the 5 replicate UU, UC and CU populations, after three days of high (150 flies/vial) or low (8 flies/vial) density conditioning.

mental or maternal effects. Eggs were collected from the adults of each stock population and placed in 8 dram vials at low densities of 60–80 eggs per vial. Eclosing adults from these vials were then collected into cages; eggs laid by these adults were collected and transferred into 8 dram vials at low densities of 60–80 eggs per vial. Adult flies eclosing in these vials were collected one day after eclosion, and subjected to one of the two conditioning treatments: crowded (75 males and 75 females per vial) or uncrowded (4 males and 4 females per vial). All conditioning vials contained exactly 5 ml of food medium. For each population, 7 vials were set up at high density and 15 at low density, adding up to a total of 330 conditioning vials (3 selection regimes × 5 replicate populations × (7 vials at high density, 15 vials at low density). The flies remained in these vials for 3 days, after which they were used for setting up the longevity assay described below.

Longevity assay

After 3-day conditioning period, flies were placed into vials with about 3 ml of food medium at a density of 4 males and 4 females per vial; 10 such vials were set up for each population × conditioning combination. Thus, a total of 2400 flies were assayed for longevity. The flies were transferred to fresh food vials every third day until all flies had died. All vials were checked daily for deaths. Dead flies in a vial were not replaced over the course of the assay. Life-span was measured as the time, in days, from the start of the conditioning to the day of death. The longevities reported here are, consequently, the conditional longevities of flies that survived the 3 days of conditioning.

Statistical analysis

The longevity data were subjected to an analysis of variance (ANOVA) using the procedure GLM of SAS for Windows version 6.08. Due to the pattern of relatedness among the CU, UC, and UU populations (CU_i ,UC_i ,and UU_i are more closely related to each other than either of them is to other populations with which they share the same selection regime, $i = 1..5$), sets of CU, UC and UU populations, matched by subscripted indices, were treated as random blocks in the analyses. Selection-

regime and conditioning density were treated as fixed effects crossed within each block (our primary interest was in these fixed effects; block in this experiment confounds ancestry and handling effects and, thus, although we are interested in accounting for this variation by treating block as a factor, we do not try to interpret any significant random effects). Because the measurement of longevity was done on individual flies in each vial, the ANOVA model included vial as a random effect nested within the block × selection × conditioning interaction. Sex was treated as a fixed factor crossed with all the rest.

From the data on longevity, we also estimated the age-independent (A) and age-dependent (α) parameters of the Gompertz equation that models the age-dependence of mortality rates[28], using a maximum likelihood method utilizing untransformed survival data[31]. The estimates of A and α from each population ´ conditioning combination were then used as data for analyses of variance, treating selection-regime, conditioning and sex as fixed factors crossed within the 5 replicate blocks. In similar previous experiments we have tested the goodness of fit of the Gompertz equation to data on mortality collected from males and females of over 20 populations of *D. melanogaster* related to the populations used in this study under various environmental conditions and the fit has always been excellent with R^2 values consistently being above 90% (refs 17,31).

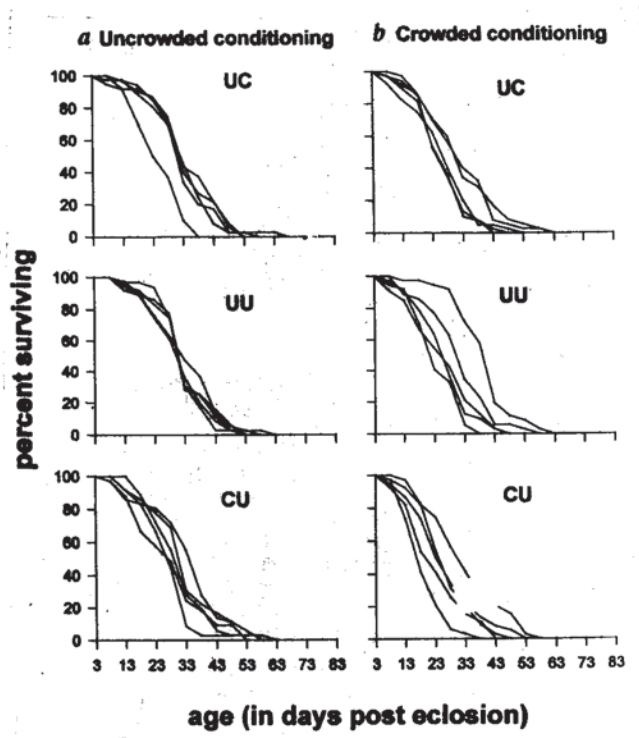

Figure 3. Per cent survival to various ages of adult females from each of the 5 replicate UU, UC and CU populations, after three days of high (150 flies/vial) or low (8 flies/vial) density conditioning.

Effect of adult crowding on longevity

The ANOVA results for longevity after 3 days of adult conditioning showed significant fixed effects of sex and conditioning density; selection regime did not have a significant effect (Table 1). In general, longevity decreased with increasing density of conditioning (an average reduction due to crowding of 2.75 days), and, as expected for *Drosophila*, males outlived females by an average of 9.2 days (Figure 1). Females were seemingly affected by crowding to a degree greater than the males: crowding reduced the longevity of females by an average of 4.2 days whereas in the case of males, the reduction was only 1.3 days.

Unfortunately, too much cannot be made of these differences because the early death of large numbers of females in the crowded treatment would tend to increase life-span in males due to reduced courtship and mating, thus counteracting to some degree the detrimental effect of the crowding treatment experienced earlier. Moreover, the degree and duration of crowding experienced by the populations in this study was moderate (mean mortality in our populations during the 3 days of crowded conditioning varied from less than 1% to 11.6%, whereas more severe crowding for 5 days in the same populations can yield mortality up to 90% (ref. 41). We were, therefore, more interested in broad patterns of mortality effects than in the specific magnitudes of the effects. Overall, there did not appear to be major differences in the pattern of effects of adult crowding on either mean longevity in the UU, UC and CU populations (Figure 1), or on the survivorship curves of males and females from these three types of populations (Figures 2, 3). The only significant difference among selection lines was that the mean longevity of UC males from the crowded conditioning treatment was greater than that of both CU ($P = 0.02$) and UU ($P = 0.01$) males. At a qualitative level, nevertheless, the per cent decline in mean longevity due to adult crowding was the greatest in the CU, and the least in the UC populations.

The ANOVA results for the age-independent (A) and age-dependent (α) mortality parameters of the Gompertz equation indicated that the crowded conditioning treatment lowered longevity through an increase in A. Of the fixed factors in the ANOVA model, sex, conditioning, and the selection regime × sex interaction were seen to have significant effects on the estimates of A, while only sex had a significant effect on estimates of α (Table 2). The reduced longevity of females, as compared to males, is well known in *Drosophila*[7,31], our re-

Table 2. Estimates of the age-independent (A) and age-dependent (α) mortality parameters of the Gompertz equation. The entries are the means (± 95 % confidence intervals) of the five replicate populations in each selection regime × conditioning combination. The only significant effects in the ANOVA were those of sex, conditioning, and selection regime × sex on estimates of A, and of sex on estimates of α.

	Uncrowded conditioning		Crowded conditioning	
Selection regime	Male	Female	Male	Female
Estimates of A				
UU	0.0026	0.0030	0.0031	0.0043
	(0.0010)	(0.0012)	(0.0015)	(0.0019)
UC	0.0027	0.0033	0.0029	0.0048
	(0.0009)	(0.0011)	(0.0017)	(0.0015)
CU	0.0025	0.0035	0.0035	0.0071
	(0.0010)	(0.0010)	(0.0008)	(0.0025)
Estimates of α				
UU	0.0876	0.1051	0.0886	0.1054
	(0.0202)	(0.0184)	(0.0232)	(0.0157)
UC	0.0807	0.1022	0.0729	0.1015
	(0.0124)	(0.0218)	(0.0190)	(0.0325)
CU	0.0863	0.0956	0.0796	0.0998
	(0.0089)	(0.0149)	(0.0158)	(0.0366)

sults show that this is due to both greater age-independent mortality (mean A in females was 0.0043, a 53% increase over the mean in males, 0.0028) and a more rapid rate of ageing (mean α in females was 0.1016, a 23% increase over the mean in males, 0.0826). The mean value of A in populations subjected to the crowded treatment was 0.0042, a 44% increase over the mean in the uncrowded treatment, 0.0029. The significant selection regime × sex interaction for estimates of A arose from the fact that means of selection regimes for the crowded and uncrowded treatments did not differ significantly in the case of males, but did so in the case of females (Table 2).

Conclusions

The results of the present study are consistent with the findings of previous studies that adult crowding reduces longevity in *Drosophila*[33,37]. Moreover, our results indicate that even brief periods of relatively moderate crowding can have a lasting effect on subsequent longevity (Table 1, Figure 1). Interestingly, it is also clear that, like other environmental factors affecting longevity in *Drosophila*[17,32], adult crowding affects longevity by altering the age-independent mortality rate rather than the rate of ageing *per se* (Table 2). The commonly observed difference in longevity between males and females in *Drosophila* is shown to be due to increased age-independent mortality, as well as an increased rate of ageing in females (Table 2).

Although selection regime did not have a statistically significant effect on the extent to which crowding reduced longevity (Table 1), our results suggest that the UC populations may, in fact, have a reduced sensitivity to adult crowding, as a result of having been subjected to high adult densities for over 50 generations (Figure 1). Possibly, a more severe bout of crowding would lead to differences among the selected lines that would be picked up as being statistically significant. This interpretation is supported by the finding that the UC populations are significantly less sensitive to adult crowding effects on fecundity and mortality during crowding than either the CU or the UU populations[41].

One very consistent finding of previous studies on environmental and genetic modifications of longevity has been the existence of a ubiquitous trade-off between female fecundity and longevity[2,3,17]. This trade-off has been interpreted in light of the so-called Y-model of resource allocation[6,10,42]. In this view, reproduction and survival are envisioned as being alternative candidates for the allocation of energy reserves by organisms, such that, all other things being equal, increased reproductive output comes at the expense of survival, and *vice versa*. A series of studies on *Drosophila* populations closely related to our CU, UC and UU lines, suggest that fecundity and longevity in these populations are, in fact, linked in this manner, based upon their common dependency on lipid as a resource[4,6,27,43]. Similarly, most environmental factors increasing or decreasing longevity in many species have been shown to do so by respectively reducing or enhancing fecundity. Since the reduction of female fe-

cundity by adult crowding is a well-documented phenomenon in *Drosophila*[44], one might naively expect that adult crowding would, therefore, enhance longevity.

In fact, the observed reduction in longevity, by levels of adult crowding that are known to also depress fecundity in the UU, UC and CU populations[41], strongly suggests that the negative impact of adult crowding on longevity in our experiment is mediated by a reduction in the overall levels of stored energy reserves in the flies subjected to the crowded conditioning treatment. In this context, it is interesting to note that even 24 hours of adult crowding reduced subsequent starvation resistance in the B populations from which our experimental populations were derived[37]. Given the close relationship between starvation resistance and lipid content in *Drosophila*[4,6,8], this suggests that brief episodes of adult crowding may significantly deplete lipid reserves. However, there are also indications that reserves of glycogen, rather than lipid, may be more severely affected by adult crowding[37]. Evidently, further studies of the physiological consequences of adult crowding are needed to obtain a clearer picture of how the effects of crowding on adult fitness components are mediated by the underlying architecture of inter-related metabolic pathways.

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