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Influence of two methods of dietary restriction on life history features and aging of the cricket *Acheta domesticus*

Janice Christina Lyn · Wida Naikkhwah · Vadim Aksenov · C. David Rollo

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Abstract Studying aging is constrained using vertebrates by their longevity, size, ethical restrictions, and expense. The key insect model, *Drosophila melanogaster*, is holometabolous. Larvae feed on yeast in moist media and adults sponge food. Most aging studies are restricted to adults. Another key model, the nematode *Caenorhabditis elegans*, feeds on bacteria in moist media. For either invertebrate refreshing test materials, preventing degradation and obtaining accurate dosing are difficult even with synthetic media. The cricket *Acheta domesticus* has a short lifespan (~120 days at 30°C) and is omnivorous. Age-matched cohorts are easily obtained from eggs. The life cycle is hemimetabolous and nymphs eat the same foods as adults. Growth is easily monitored, gender can be differentiated before maturity, and maturation is indicated by wings and mature genitalia. Crickets can be reared in large numbers at low cost. Test materials can be mixed into food and ingestion rates or mass budgets easily assessed. Here, we validate the cricket as a model of aging by testing two fundamental methods of restricting food intake: time-restricted access to food and dietary dilution. Growth, maturation, survivorship, and longevity varied with treatments and genders. Intermittent feeding (which is ineffective in flies) significantly extended longevity of crickets. Dietary dilution also extended longevity via remarkable prolongation of the juvenile period.

Keywords *Acheta domesticus* · Aging · Dietary restriction · Growth · Maturation · Compensation

J. C. Lyn · W. Naikkhwah · V. Aksenov · C. D. Rollo (✉)
Department of Biology, McMaster University,
1280 Main St. West,
Hamilton, Ontario, Canada L8S 4K1
e-mail: rollocd@mcmaster.ca

Introduction

Mice and rats are key models for aging research but are disadvantaged by their relatively large size and long lifespan. Escalating ethical restrictions and expense restrict sample sizes and statistical power. Testing dietary interventions for impacts on aging takes years. Although vertebrates and invertebrates differ greatly in morphology, their cell physiology, biochemistry, metabolism, and nutrition share great similarity. Genes and signaling pathways associated with aging, and even some human diseases, are phylogenetically conserved (Partridge and Tower 2007; Mair and Dillin 2008; Shaw et al. 2008).

Crickets (*Acheta domesticus)* are ideal for studying nutrition and aging since they are omnivorous and live only ~120 days at 30°C. Nymphs and adults have similar requirements. Large cohorts of known age can be generated from eggs, growth is easily monitored and gender can be discerned at young ages. Reproductive effort of both females (oviposition) and males (singing) can be quantified. Maturation is readily

detected by expression of wings and adult genitalia. Raising large numbers of crickets entails little space or cost. Test materials can be mixed into food and ingestion or mass budgets can be readily derived. Adult crickets have neuronal replacement in brain (Cayre et al. 1996) and they are a model for developmental biology amenable to interference RNA studies. Nymphs have strong regenerative capacity, a topic also of interest for aging (Mito and Noji 2008; Nakamura et al. 2008).

Drosophila and *Caenorhabditis elegans* have greatly contributed to identifying genes, pathways and mechanisms involved in aging, partly because of their short lives and ease of production (Partridge and Tower 2007). Other advantages include availability of mutations, genetically modified lines and genome maps. Both species, however, have drawbacks for testing supplements or drugs. *Drosophila* is holometabolous and larvae feed on yeast in moist media while adults sponge food. *Caenorhabditis elegans* feeds on bacteria in moist cultures. Thus, refreshing materials or accurate dosing are difficult even with synthetic media (but see Ja et al. 2007).

Interactive aspects of growth, stress responses, energy metabolism and longevity are regulated by the insulin/IGF-1-PI3K signaling pathway (with extensions to FOXO, sirtuins, and TOR) (Partridge and Tower 2007; Mair and Dillin 2008; Rollo 2010a, b). Most models of extended longevity, including dietary restriction (DR), show alterations in these elements. DR involves reducing food below ad libitum while maintaining micronutrients, although recent theory highlights protein/carbohydrate balance (Weindruch and Walford 1988; Finch 2007; Everitt et al. 2010; Simpson and Raubenheimer 2010). Features expressed in DR (e.g., slow growth, delayed maturation and stress resistance) likely represent adaptive plasticity (Holliday 1989; Rollo 1994). This is supported by studies with *C. elegans* and *Drosophila* where even food odors alter longevity and reduce gains from DR (Alcedo and Kenyon 2004; Libert et al. 2007; Partridge and Tower 2007).

DR of flies is generally achieved by diluting nutrients or yeast and most studies use only adults (Chippindale et al. 1993; Partridge et al. 2005; Min and Tatar 2006; Piper and Partridge 2007; Tatar 2007; Lee et al. 2008). Min et al. (2007) found that caloric intake by flies was reduced on all diets that extended longevity but yeast concentration explained DR responses better than sugar or calories (Mair et al. 2005). *Drosophila* may have nutritional, olfactory and feeding specializations for yeast (Mair et al. 2005; Min et al. 2007; Partridge and Tower 2007) that could complicate dietary manipulations or testing supplements (Le Bourg and Minois 2005). *Acheta domesticus* is an unspecialized omnivore with direct development, properties amenable to applying dietary restriction in youth (when greatest benefits of DR accrue in vertebrates).

Most species express compensatory feeding on diluted diets or increased feeding and growth following periods of insufficiency (see Wang et al. 2006). Compensation, however, has costs that can reduce lifespan (Metcalfe and Monaghan 2001, 2003). DR of *Drosophila* may not be comparable to vertebrates if DD yields compensation. Whether life extension of DR *Drosophila* involves alterations in aging rates as opposed to acute survivorship is another critical question (Mair et al. 2003; Partridge et al. 2005; Burger et al. 2007; Tatar 2007). Several studies with flies failed to obtain life extension with DR (Carey et al. 2002; Cooper et al. 2004; Le Bourg and Minois 2005). *Drosophila* normally search widely for temporary resources so it has been suggested that DR of flies is more likely to induce dispersal rather than slow aging (Le Bourg and Minois 2005). Ja et al. (2009) suggest that many DR studies with *Drosophila* actually reflect water deprivation.

We examined impacts of DD (with cellulose) versus DR (intermittent access to food) to assess these methods and suitability of *A. domesticus* as a model for aging. We hypothesized that crickets would show life extension when dietary restriction was applied in youth. We also expected to see major compensatory adjustments, not just in feeding, but also life history features. We employed two levels of intermittent feeding. Interest in alternate day fasting in vertebrates is growing because humans can maintain this regimen and because the mechanism deriving benefits may involve alternating stimulation of TOR and FOXO (Rollo 2010a, b). We expected that crickets might provide a good invertebrate model as their lower metabolic rate might better tolerate this approach whereas *Drosophila* cannot. We further examined various levels of dietary dilution because it is applied in some vertebrate studies, nematodes and virtually all work with *Drosophila.*

Methods

Breeding colony A colony of *A. domesticus* was established with genetically heterogenous stock. Crickets were housed in an acrylic enclosure (93× 64.2×46.6 cm) with egg carton shelters. The top was covered with 1 mm^2 plastic mesh that prevented escape while providing ventilation. The enclosure was encased in 1.5-cm-thick Durofoam® insulation. Chicken feed (Quick Feeds©) fresh carrots and dechlorinated water (delivered via a soaked cellulose sponge) were provided ad libitum. Pro-mix® general-purpose soil in shallow trays served as oviposition medium. A 1-mm^2 plastic screen over the soil allowed oviposition but prevented digging. The soil was sprayed with dechlorinated water daily. The colony was maintained at 30±1°C with a 12 h light/12 h dark photoperiod.

Hatchlings Egg-laden soil was transferred to plastic containers and covered with egg cartons to facilitate harvesting hatchlings (incubation: ~10 days). Water was provided ad libitum. Containers were sealed with plastic wrap with pinhole perforations to maintain humidity and ventilation. Insect Gutload© (Repashy Superfoods) served as standard diet (protein 20%; fat 4.5%; fiber 10%; calcium 8%; phosphorous 0.5%; vitamin D3 20 IU/g; vitamin A 200 IU/g; beta carotene 5 mg/g; choline 6 mg/g; vitamin C 2.5 mg/g; vitamin E 0.1 mg/g; B vitamins (B1, B2, B3, B5, B6, B7, B12) 0.83 mg/g; vitamin K 0.03 mg/g). Ingredients: Alfalfa meal, wheat germ, hempseed meal, brewer's yeast, bee pollen, whey protein, calcium carbonate, fig powder, diacalcium phosphate, algae (spirulina, kelp, haemotococcus), plant extracts. Carbohydrate and caloric content are unknown.

Dietary restriction DR may be achieved by providing measured amounts of food or restricting feeding to specific times. Intermittent feeding was most feasible for large cohorts of animals. Crickets were randomly assigned to groups of 10 housed in 15 cm^3 transparent plastic containers. Holes (1 mm) in the lids provided ventilation. Each container contained an egg carton shelter, food dish and a microtube that delivered distilled water via a cellulose sponge plug. Containers were housed in enclosures with conditions similar to the breeding colony. Sixty hatchlings were fed ad libitum. Sixty crickets had access to food for only 12 h separated by 24 h intervals (DR24). A "severe" DR regime (60 crickets) had access to food for 12 h every 36 h (DR36). DR was initiated at 6 days of age. All groups were weighed weekly and mortality was assessed daily. Gender was apparent as ovipositors became apparent at ~40–50 days. Genders were separated before sexual maturity. Random samples of 10 crickets from each group were weighed weekly. We only detected four instances of cannibalism and no coprophagy under DR. These were excluded from analyses.

Feeding under DR was not monitored in experimental animals but was separately analyzed in 30 newly mature crickets (15 of each gender) placed on each of the three treatments. Crickets were acclimated to experimental conditions for 2 days with ad libitum access to Insect Gutload© and distilled water. Individual crickets were weighed and randomly assigned to treatments (DRC, DR24, or DR36). Food was provided according to the schedules in the lifetime studies. Food was pre-weighed and a sample was taken to estimate original water content (~2%). Uneaten food was removed after 12 h and dried to constant weight. Consumption was calculated by subtracting the dry weight of uneaten food from the estimated dry weight of original food. Values were divided by the live mass of crickets to obtain mass-specific values. Compensation was assessed by comparing only the 12 h feeding periods among treatments across 6 days. Average feeding per treatment was calculated including those periods where no food was available (e.g., DR36 had only three 12 h feeding periods over 6 days).

Dietary dilution Diets were diluted by adding α-cellulose (Sigma) to Gutload©. Cellulose is routinely used to dilute insect diets (Lee et al. 2004) although some digestion was detected in cockroaches (Jones and Raubenheimer 2001). Thirty nymphs (14 days old) were randomly assigned to each of five dilutions: 0% (DDC), 25% (DD25), 40% (DD40), 55% (DD55), and 75% (DD75). Housing and maintenance were the same as for DR. Diets were prepared weekly and refrigerated. All diets contained 0.9 g of agar dissolved in 90.0 ml of distilled water at 40°C. Appropriate amounts of "Insect Gutload"© and cellulose were mixed to obtain homogenous solutions that were then set in a refrigerator: DDC (no cellulose, 6.3 g of food), DD25 (1.58 g cellulose, 4.73 g food),

DD40 (2.52 g cellulose, 3.78 g food), DD55 (3.47 g cellulose, 2.84 g food), and DD75 (4.73 g cellulose, 1.58 g food). Once firm, diets were dried at 40°C to constant weight and ground to a fine powder. Preliminary observations indicated that powdered diet supported better growth and survival. Dishes with an opening on one side prevented spillage of ad libitum food. Food was pre- and post-weighed to quantify consumption of experimental animals among treatments. Random samples of 10 crickets from each treatment were weighed weekly. Eight instances of possible cannibalism were observed for DD nymphs.

Data analysis ANOVA and ANCOVA were applied for various yield variables (growth rates, maturation age and mass, feeding rates, and longevities [mean, 10%, maximum] [significance level of $p<0.05$]). Newman−Keuls resolved differences among groups. Most analyses employed Excel® or Statistica®. Survivorship analyses describe cumulative population losses. Kaplan−Meier log-ranked survival estimation was used to analyse the distribution of survival. Gehan's Wilcoxon tests assessed differences between groups.

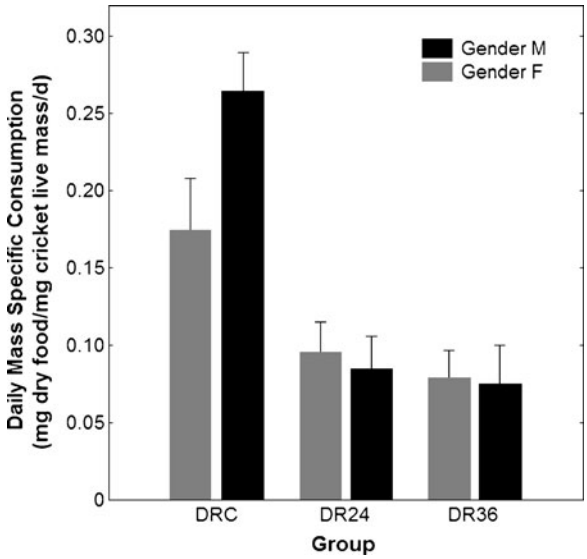

Fig. 1 Mean mass-specific consumption for *A. domesticus* on dietary restricted diets (milligrams dry food/cricket live weight). ANOVA of average consumption for mature crickets found highly significant differences among groups ($p<0.00001$). Both restricted groups differed significantly from controls (Newman−Keuls, both $p<0.001$)

Results

Feeding For mature crickets, ANOVA detected significant impacts of DR on mass-specific consumption ($p<0.00002$). Newman−Keuls resolved that both DR24 and DR36 differed from DRC (both $p<0.0001$). Intake on DR24 was 42% that of DRC and DR36 intake averaged only 37% that of DRC (Fig. 1). Gender had no impact on mass-specific feeding ($p>0.26$). We further examined compensatory feeding by comparing intake limited to the 12 h periods when food was available. DR24 crickets ate 125% that of DRC and intake of DR36 animals was 148% of controls. Despite these trends ANOVA obtained only marginal significance among treatments ($p>0.08$). Pooling DR treatments and performing a one-way *t* test resolved significant increases in food intake when food was available under DR ($p<0.005$). Gender remained non-significant ($p>0.25$).

ANOVA detected highly significant impacts of DD ($p<0.00001$) on mass-specific feeding (Fig. 2). High mortality of younger crickets suggested they were stressed more than older crickets so we examined feeding of animals ≤50 days old (from hatching) or >50 days of age. All DD groups and ages differed from respective DDC (Newman−Keuls, all $p<0.01$). For DDC and DD25, older animals ate ~12% less than younger crickets, likely reflecting lower metabolism. Note that crickets younger than 50 days of age were nearly exclusively nymphs whereas older crickets were late instar nymphs and adults. Both young and older DD25 crickets ate ~1.6 times more than controls. At greater dilutions, however, young animals did not increase compensation further (feeding was ~1.6-fold greater than respective controls for either DD40 or DD55 groups). In contrast, older crickets on DD40 and DD55 increased feeding by 1.9- and 3.4-fold, respectively (Fig. 2).

Survivorship Kaplan−Meier survivorship analysis resolved significant impacts of DR on crickets (Fig. 3a, Table 1). Gehan's Wilcoxon tests detected differences among all three groups ($p<0.003$). ANOVA detected significant differences in mean longevity ($p<0.0001$, Table 1). The upper 95% confidence interval for DR24 (Table 1) was 28.5% greater than DRC whereas that for DR36 was 31.2% *less* due to high early

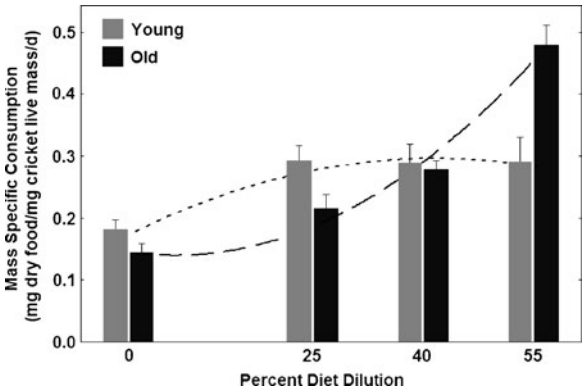

Fig. 2 Mean mass-specific consumption of young versus older *A. domesticus* on dietary diluted diets (milligrams dry food/cricket live weight). ANCOVA (with age as covariate) of weekly consumption (milligrams dry food/milligrams live mass) found highly significant differences among groups ($p<0.00001$). Compensation for DD differed greatly between young (<50 days old) and older (>50 days old) crickets. Young DD25 crickets ate ~1.6 times more than DDC (mass specific) but compensation did not increase with further dilution. In contrast, older crickets increased feeding by 1.9- and 3.4-fold on DD40 and DD55, respectively. *Single asterisk* indicates difference compared to respective control (i.e., young or old) (ANOVA, Newman−Keuls $p<0.05$). *Double asterisks* indicate ANOVA, Newman−Keuls $p<0.0005$. Age group: old: second order polynomial regression: $y=-0.000007X^2+0.0058X+0.1834$; $r=0.605$, $p<0.05$. Age group: young: second order polynomial regression: $y=0.0001X^2-0.0011X+0.1479$; $r=0.871$, $p<0.00001$

mortality. DR24 and DR36 females reached maximal longevities 128% and 140% greater than DRC, respectively. DR24 and DR36 males obtained respective increases in maximal longevity of 123% and 118%. Besides smaller increases in maximal longevity, males had lower early survivorship than females. By 70 days of age, 71% of crickets on DR36 were female. ANOVA of the last 10% surviving detected a strong treatment effect ($p<0.001$) but no impact of gender ($p>0.05$). The last 10% of surviving DR24 males and females lived 132% and 136% longer than DRC, respectively. Although DR36 exhibited greatest maximal longevity (Table 1), 70% of juveniles died by ~30 days (Fig. 3a). The last 10% of surviving DR36 males and females obtained 103% and 134% extension of lifespan relative to DRC.

Kaplan−Meier survivorship analysis found significant impacts of DD (Fig. 3b). Gehan's Wilcoxon tests differentiated DDC versus DD40 ($p<0.01$) and DDC from DD55 ($p<0.00001$). Early survivorship declined more steeply with increasing levels of DD (Fig. 3b). At 40 days all DDC were alive but DD25, DD40 and DD55 showed losses of 20%, 15% and 25%, respectively (Fig. 3b). All DD55 crickets died by 93 days. Mean longevity varied strongly with DD (ANOVA, $p<0.000001$, Table 1). Survivorship on

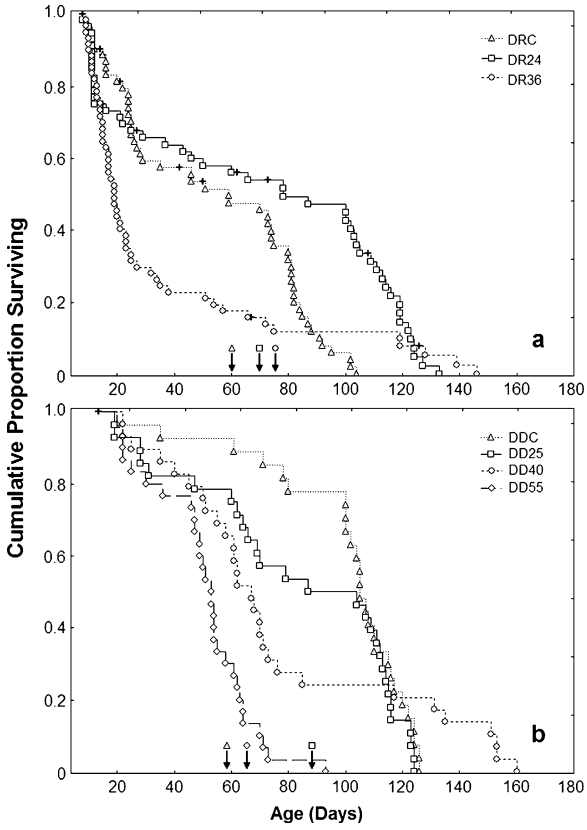

Fig. 3 Survivorship curve of dietary restricted (*a*) and dietary diluted (*b*) *A. domesticus*. **a** Groups of 60 crickets were deprived of food for periods of 24 h (DR 24) or 36 h (DR 36) (with unlimited access to water). Deprivation was followed by 12 h access to ad libitum food and water. ANOVA detected a significant difference among mean longevity of DR 24, DR 36, and control groups ($p<0.0001$). Gehan's Wilcoxon tests revealed significant differences between control and DR 24 groups ($p<0.0001$), control and DR 36 groups ($p<0.00014$), and DR 24 and DR 36 groups ($p<0.003$). **b** Crickets were assigned to increasing levels of dietary dilution (0% (control), 25%, 40%, and 55%) (with unlimited access to water) with 30 crickets per dilution treatment. ANOVA revealed a significant difference between the mean longevity of control and dietary diluted 25%, 40%, and 55% treatments ($p<0.000001$). Gehan's Wilcoxon Test found significant differences between control and dietary diluted 40% ($p<0.0112$) and control and dietary diluted 55% ($p<0.00001$). Average maturation times for each group are indicated on the X axis (by *arrows*)

Table 1 Means, upper 95% confidence interval (CI) and maximal longevities of *A. domesticus* subjected to dietary restriction and dietary dilution

Group	Mean longevity	Percent	Upper 95% CI	Maximal longevity (days)	Percent
DRC	52.1		60.9	104	
DRC F				104	
DRC M				101	
DR24	64.6	128.5	78.2	133	128
DR24 F				133	128
DR24 M				124	123
DR36	32.6	68.8	41.9	146	140
DR36 F				146	140
DR36 M				119	118
DDC	99.6		110.2	126	
DDC F				126	
DDC M				124	
DD25	83.6	83	97.6	124	98
DD25 F				124	98
DD25 M				124	100
DD40	76.9	77	92.7	164	130
DD40 F				126	100
DD40 M				164	130
DD55	50.6	50	57.1	93	75
DD55 F				69	55
DD55 M				93	75

DD25, DD40 and DD55 showed reductions of 16.4% ($p>0.06$), 23.1% ($p<0.006$) and 49.4% ($p<0.0001$) respective to DDC (Newman−Keuls). ANOVA of the last 10% surviving was also significant ($p<0.00001$), those on DD25, DD40 and DD55 obtaining means 98.3%, 120.8%, and 52.3% that of DDC. Newman−Keuls detected differences in age at death for the last 10% surviving for DDC versus DD55, DD55 versus DD25, and DD55 versus DD40 ($p<0.0002$ for all). Upper 95% confidence intervals indicated that DD25, DD40, and DD55 had 11.4%, 15.9%, and 48.2% lower survivorship than DDC, respectively, mainly reflecting juvenile mortality (Table 1). Interestingly, DDC and DD25 survivorship converged at 100 days and both groups obtained maximal longevities of 125 days (Fig. 3b). DD40 intersected DDC at ~120 days but maximal longevity on DD40 reached 164 days. The oldest DD40 female died at 126 days (similar to controls) and further life extension to 164 days was confined to 12 males representing the last 20% of the original population (Fig. 3). Less than half of these males ever matured. Males also lived longer than females on DD55. At 57 days there were eight remaining males but only one female. Maximal female survival on DD55 was 69 days compared to 93 days for males. Thus, converse to DR, DD males survived better than females and only DD40 males showed extended lifespan.

Growth Dividing maturation mass by maturation age estimates growth rate across the juvenile phase (Table 2: milligrams/day). Only individuals that reached maturity were included. ANOVA detected significant impacts of DR on juvenile growth ($p<0.05$). Newman−Keuls differentiated DRC from DR24 and DR36 (both $p<0.03$). Juvenile growth strongly varied with gender (ANOVA, $p<0.0002$). DRC females grew 1.8 times faster than males (milligrams/day, Table 2). There was a trend for reduced gender size dichotomy in DR treatments (Table 2). DR24 females grew 32% slower than DRC females (n.s.) whereas DR36 females grew only 13% slower than DRC (n.s.). For males, DR24 and DR36 groups expressed only 5% and 13% slower growth than DRC, respectively (n.s.). Thus, juvenile growth showed strong compensation other than for DR24 females (Table 2). ANOVA also detected strong impacts of DD ($p<0.0000001$) and gender ($p<0.002$)

Table 2 Impacts of dietary restriction and dilution on maturation mass, maturation age, and growth rates of crickets

Treatment	Sex	Maturation mass (mean ± SE, mg)	Percent	Maturation age (mean ± SE, days)	Percent	Growth rate (mg/day)	Percent
1 DRC	F	254.4±15.3		58.9±1.3^{3,4,6}		4.32±1.5^{2,4,6}	
2	M	150.3±22.3		63.5±1.9^{6}		2.37±11.5^{1}	
3 DR24	F	202.7±21.0	79.7	68.8±1.8^{1,6}	116.8	2.95±11.5	68.3
4	M	159.2±21.0	105.9	70.3±1.8^{1,6}	110.7	2.26±11.5^{1}	95.4
5 DR36	F	247.0±36.4	97.1	66.0±3.2^{6}	112.1	3.74±11.5	86.6
6	M	175.5±31.5	116.8	85.3±2.7^{1,2,3,4,5}	134.3	2.06±11.5^{1}	86.9
A DDC	F	207.4±9.0^{F}		56.6±1.7^{D,E,F}		3.7±0.2^{C,D,E,F}	
B	M	185.4±6.7^{F}		59.3±1.3^{D,E,F}		3.1±0.1^{D,E,F}	
C DD25	F	162.9±10.1^{F}	78.5	63.3±1.9^{E,F}	112	2.6±0.2^{A,F}	70.2
D	M	165.0±11.6^{F}	88.9	67.5±2.2^{A,B,E,F}	114	2.4±0.2^{A,B,F}	77.4
E DD40	F	177.0±16.5^{F}	85.3	82.3±3.1^{A,B,C,D,F}	145	2.2±0.3^{A,B,E}	59.5
F	M	116.5±14.3^{A,B,C,D,E}	62.8	98.0±2.7^{A,B,C,D,E}	165	1.2±0.2^{A,B,C,D,E}	38.7

Percent values were calculated relative to respective controls. Superscripts indicate treatments differing significantly (Newman−Keuls $p<0.05$)

on juvenile growth. As in DR, females grew faster than males (Table 2). Little compensation was evident under DD and this was reflected by greater reductions in growth by DD than by DR (Table 2). The longest-lived DD40 males had the slowest growth (38.7% that of DDC males, Table 2).

Maturation mass ANOVA detected significant effects of diet ($p<0.00001$) and gender ($p<0.0001$) on maturation mass. A significant treatment*gender interaction was also resolved ($p<0.01$). Mature females were 1.69, 1.28, and 1.40 times larger than males in DRC, DR24, and DR36, respectively (Table 2, Fig. 4a). When compared to DRC females, DR24 and DR36 females were ~20% and ~3% smaller, respectively. This was also reflected in a negative relationship between maturation mass and maturation age (that was delayed by treatments) for pooled female DR data (Fig. 4a). However, only three DR36 females survived to maturity and all were relatively small. Although females showed some compensation in maturation mass, defence of maturation age appeared a higher priority (see Figs. 4a and 5a and Table 2). Surprisingly, DR24 and DR36 males were 1.06 and 1.17 times *larger* at maturity than DRC males, respectively. This was associated with slower growth but delayed maturation (Table 2). Defence of body mass by DR males was also reflected by a trend for increasing size with maturation age (Fig. 4a).

ANOVA found significant effects of DD ($p<0.0001$) gender ($p<0.008$) and a marginal treatment*gender interaction ($p>0.07$) on maturation mass (Table 2). Gender differences in maturation size were less pronounced on DD than DR (females were 1.12, 0.98, and 1.52 times the size of males on DDC, DD25, and DD40, respectively). Maturation mass declined with DD in both genders. Females on DD25 were 78.5% of DDC females indicating little compensation, whereas males maintained a mass ~89% that of DDC males (Table 2). DD40 males were smallest (116 mg) and close to expected size for the degree of DD (62.8% of DDC). This contrasts with DD40 females that were ~85% the mass of respective DDC females (Table 2).

Maturation mass of females for pooled DD data showed little relationship to maturation age, perhaps reflecting defence of maturation age (Fig. 4b). For DD males there was a clear negative relationship between maturation mass and maturation age for pooled data (Fig. 4b). Males tended to mature later than females, however (Fig. 5b), suggesting that body size was not defended even with greatly delayed maturation (and extended longevity).

Maturation age DR and DD treatments significantly delayed maturation (ANOVA, DR: $p<0.00001$, DD: $p<0.000001$, Table 2). Maturation age varied with gender (DR: $p<0.00001$, DD: $p<0.0002$) and a treatment*gender interaction was detected for DR

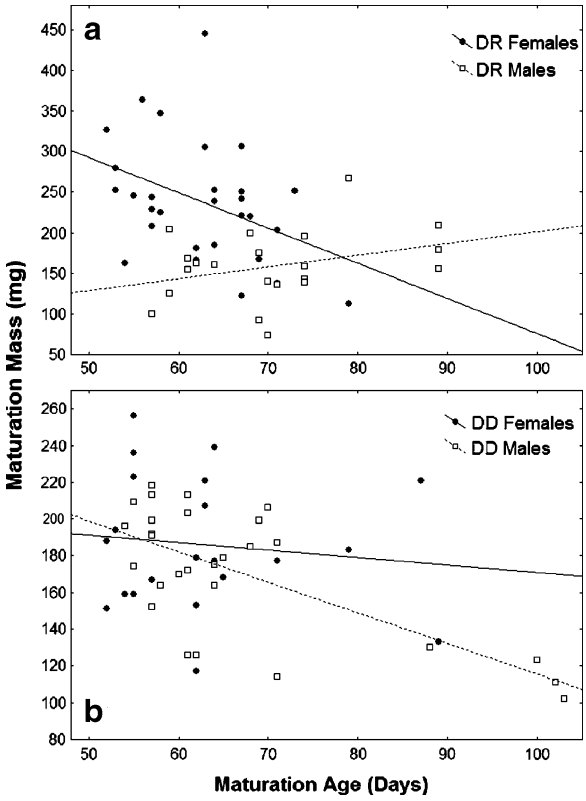

Fig. 4 Relationship of maturation mass to maturation age of *A. domesticus* for pooled data for (*a*) dietary restriction or (*b*) dietary dilution. **a** For DR females, the relationship was significantly negative ($p<0.03$) indicating that late maturing females are smaller: regression line: maturation mass=509.9689–4.3455 (maturation age), $r^2=0.1591$, $p>0.16$. DR males tended to a positive relationship ($r^2=0.1055$, $p>0.15$) indicating that late maturing males were larger. (**b**) For DD, female maturation mass and age were not strongly linked ($r^2=0.0144$, $p>0.6$) reflecting that females defended mature body size. For males the relationship was significantly negative reflecting that late-maturing males were smaller: regression analysis: maturation mass=282.79–1.6766 (maturation age), $r^2=0.4585$, $p<0.00008$. Males generally matured later than females. Most of the relationship for males traced to those maturing very late on the 40% dilution treatment. No crickets matured on the 55% diluted diet

(DR: $p<0.009$, DD: n.s). Statistical resolution of diet and gender were greater for maturation age than maturation mass suggesting age as more critical. Indeed, maturation was constrained to a relatively narrow age range (Fig. 5a, b). Other than for DR36 and DD40, maturation age varied by only 14 days (56–70 days old) among treatments. DRC and DDC crickets matured at similar ages (58.9 and 56.6 days for respective females, 63.5 and 59.3 days for respective males) (Table 2).

Females on DR24 and DR36 only took ~1.17 ($p<0.02$) and 1.12 times ($p>0.07$) longer to mature than DRC, respectively (Table 2). DRC and DR24 males also matured at a relatively similar age (n.s.). DR36 males, however, took 1.34 times longer to mature than DRC males ($p<0.0001$). Females matured earlier across DR treatments (58–69 days) than

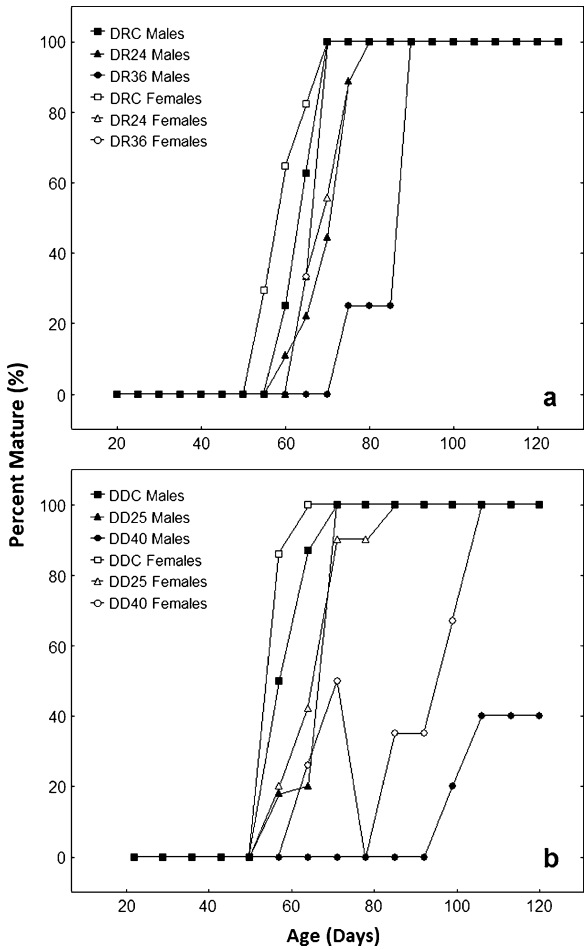

Fig. 5 Maturation schedules for (*a*) dietary restricted and (*b*) dietary diluted treatment of *A. domesticus*. **a** Maturation occurred in a relatively narrow window (48–64 days) for the control group, with females generally maturing earlier. Dietary restriction delayed maturation. Interestingly, although DR 24 individuals began to mature earlier than DR 36 individuals, DR 36 females completed maturation within the same time frame as DR 24 individuals. Males of the DR 36 treatment matured very late and many individuals died before maturing. **b** With modest or no dilution, maturation occurred in a relatively narrow window (50–65 days) with females generally maturing earlier. With 40% dilution, females tended to die shortly after maturing, suggestive of an associated cost. Males on this treatment matured very late and only 40% matured. The longest lived animals on this treatment were all immature males

DR males (63–85 days) (Table 2). The breadth of the maturation window only increased from 17 days for DRC to 22 days on DR36 (Fig. 5a).

DD25 males and females matured only 12–14% later than DDC, indicative of strong compensation. For DD40, female maturation was 45% later than DDC suggesting little compensation but DD40 males matured 65% later than DDC indicative of stress or commitment to further growth (Table 2). DD40 females showed delayed maturation compared to DDC but matured ~30 days earlier than DD40 males (Fig. 5b). This reinforces that females prioritize early maturation more than males. However, DD40 females tended to die shortly after the adult moult (Fig. 5b). First maturation was 52, 55, and 71 days for DDC, DD25, and DD40, respectively, similar to DR (52, 59, and 67 days for DRC, DR24, and DR36 respectively). Last maturation had a wider range: 70 days for DDC males but a remarkable 103 days for DD40 males (several DD40 males died at 164 days without maturing)

Discussion

Feeding, dietary restriction, and compensation Intermittent or low quality food can induce compensatory feeding or "catch up" growth (Metcalfe and Monaghan 2001, 2003). Compensation occurs in diverse invertebrates (Rollo and Hawryluk 1988; Surbey and Rollo 1991; Lee et al. 2004; Dmitriew and Rowe 2007; Morehouse and Rutowski 2010) including orthopterans (Rollo 1984; Yang and Joern 1994; Jones and Raubenheimer 2001; Berner et al. 2005; Raubenheimer and Jones 2006). Compensatory feeding can buffer growth and maturation but can incur costs in survival and reproduction (Metcalfe and Monaghan 2001, 2003).

Whether *Drosophila* compensate for diluted diets remains controversial (e.g., Carvalho et al. 2005; Partridge et al. 2005; Min and Tatar 2006; Min et al. 2007; Tatar 2007). Bross et al. (2005) suggested adult *Drosophila* show no compensation whereas Carvalho et al. (2005) showed that net nutrients declined with dilution despite increased feeding and food processing. Lee et al. (2008) found that flies compensated and could select a balanced diet. Fanson et al. (2009) found strong compensation for dilution in Queensland fruit flies. Many feeding specialists require specific cues from authentic hosts. *Drosophila* is adapted to yeast and potential compensation could be offset by dilution of gustatory cues (see Mair et al. 2005; Min and Tatar 2006; Min et al. 2007).

Intermittent feeding of crickets significantly reduced overall intake despite compensation during periods of food availability. Average daily intakes on DR24 and DR36 were only 48% and 31% that of DRC, respectively (both $p<0.0001$, Fig. 1). Compensatory increases during periods of available food ranged from 126–163% but gender and treatment were not statistically resolved. Compensation was resolved for pooled data for DR24 and DR36 compared to DRC ($p<0.005$, one-tailed t test).

Vertebrate DR may involve measured portions, alternate day fasting or DD (mainly the former two). Logistic problems have led to general use of DD to calorically restrict *Drosophila* or *C. elegans*. Intermittent feeding is difficult to apply since maggots and nematodes live in moist media. Intermittent feeding of adult flies is also ineffective, probably because high metabolic demands require frequent feeding. Mortality was ameliorated by sugar (see Carey et al. 2002; Partridge et al. 2005; Piper and Partridge 2007). Longevity was reduced rather than extended by dietary restriction of houseflies, and this was modulated by sucrose (Cooper et al. 2004). We are exploring alternative diets to reduce juvenile mortality of crickets on DR and DD. Carbohydrate is a likely target.

Immature insects are more sensitive to inadequate diets than adults (Scriber and Slansky 1981; Dmitriew and Rowe 2007) and their requirements may differ. High protein is essential for *Drosophila* larvae but adults live weeks on sugar alone (Tettweiler et al. 2005). Some suggest that DR of larvae does not alter life span of adult *Drosophila* (e.g., Pearl 1928; Partridge et al. 2005). Lack of carryover of larval stress to adult size and function was reported in caterpillars and coccinelid beetles (Dmitriew and Rowe 2007; Morehouse and Rutowski 2010). This is consistent with theory that onset of senescence is associated with maturity (Williams 1957) but DR in vertebrates is most effective if instituted at young ages. In fish, juvenile rather than adult diet was the main determinant of most key life history features (Taborsky 2006). A novel possibility is that holometabolous life histories buffer adult phenotypes from developmental variation associated with environmental stress and nutritional. Thus, larval compensation may achieve targets that minimize variation and

maximize adult fitness (see Gotthard et al. 1994; Morehouse and Rutowski 2010). If so hemimetabolous insects may best compare to vertebrates.

The assumption that larval experience does not affect adults has resulted in most studies of flies being restricted to adults. This also differs from most vertebrate studies raising the question of whether results even reflect common mechanisms (see Grandison et al. 2009a). Indeed, some results of DR in *Drosophila* trace to water supply (Ja et al. 2009). Different methods of DR in *C. elegans* indeed induce different longevity-associated pathways (Greer and Brunet 2009). Longevity extension via both DR and DD in crickets is relevant here. Different mechanisms are suggested given that genders favored by DR or DD of crickets may differ and DD extended longevity largely by prolonging immaturity. Intermittent feeding initiated with juvenile crickets (with ad libitum water) appears comparable to vertebrate studies. Success with intermittent feeding may reflect larger size and lower metabolic rates of crickets compared to flies. Our degree of restriction (~50% to 70%) was actually greater than applied to mammals (~30–40%), suggesting more moderate restriction might reduce stress.

Compensation for DD differed greatly between young (<50 days old) and older (>50 days old) crickets. Young DD25 crickets ate ~1.6 times more than DDC (mass specific) but compensation did not increase with further dilution (Fig. 2). In contrast, older crickets increased feeding by 1.9- and 3.4-fold on DD40 and DD55, respectively (Fig. 2). Increased compensation by older animals was particularly surprising as many were larger and had finished growing so their metabolic demands might be expected to be less. Compensation fell short of the degree of dilution, however, effectively restricting actual nutrient intake. Compensatory scope is constrained by high metabolic demands (Rollo and Hawryluk 1988) in which case resource shortfalls may impose exceptional stress (Rollo 1986). Gotthard et al. (1994) found that *Pararge aegeria* caterpillars expressing faster growth lost weight faster and had greater mortality during starvation (Gotthard et al. 1994). Alternatively, nymphs may have found the high fiber diet difficult to ingest.

Whether extension of lifespan by DR even traces to reduced calories is under scrutiny (Finch 2007; Min et al. 2007). Amino acid restriction extends longevity despite compensatory increases in feeding (Rollo 2002; Piper and Partridge 2007; Archer et al. 2009). Nutritional balance varies tradeoffs among growth, reproduction, adiposity, immunity and longevity. The "nutritional geometry" paradigm suggests that actions of DR largely reflect protein/carbohydrate balance rather than reduced calories (Carey et al. 2008; Skorupa et al. 2008; Archer et al. 2009; Behmer 2009; Fanson et al. 2009; Ja et al. 2009; Simpson and Raubenheimer 2010). In *Drosophila* high carbohydrate/protein balance favors longevity whereas flies prefer diets with high protein that favors reproduction over longevity (Lee et al. 2008). Grandison et al. (2009b) found that amounts and balance of amino acids also modulate fecundity and longevity of *Drosophila*. Carbohydrate/protein balance alters lifespan of *Teleogryllus* crickets (Maklakov et al. 2008, 2009). We showed that carbohydrate supplements favorably impacted mice whereas a 38% protein diet reduced longevity and increased free radical processes (Rollo et al. 1996). Alternate day fasting may extend longevity by balanced stimulation of synthetic pathways on feeding days and stress resistance pathways on fasting days (Rollo 2010a, b).

Survivorship DR and DD generally induced high juvenile mortality followed by a phase of slower loss and finally, accelerating mortality in senescence (Fig. 3a, b). DD cohorts were started at 14 days of age, as otherwise this treatment was more stressful than DR begun at 6 days. Thus, reduced early mortality for DDC compared to DRC likely reflects the older initial ages of DD nymphs. The control diets are also not directly comparable as they differ in composition and in consistency. Besides poor compensation, juvenile mortality can trace to small size, high surface area to volume ratios, poor stress resistance or immunity, moulting, or stabilizing selection (Promislow and Harvey 1990; Albers and Bradley 2006). Slow growth protracts vulnerability. *Drosophila* studies generally ignore juvenile mortality altogether. It is possible that crickets tend to a type III survivorship curve or better juvenile survivorship may be obtained by altering diets and experimental design. Cricket survivorship curves resembled those of vertebrates and *Drosophila* if only the adult period was considered (Magwere et al. 2004). Some early vertebrate studies of DR induced relatively high juvenile mortality when initiated at weaning, but this was avoided by using older juveniles (see McDonald

and Ramsey 2010). Cricket survivorship might also be improved by using older juveniles. Regardless, other than for extreme dilution life extension was positively associated with juvenile mortality suggesting that early stress resistance may lead to extended longevity.

Deferral of senescence contributed to longevity extensions of 18% and 40% with DR24 and DR36, respectively (Fig. 3a, Table 1). After 30 days, DR24 expressed superior survivorship over other groups resulting in significant extension of mean and maximal longevity (Table 1). Exceptional loss of DR36 juveniles lowered mean longevity but maximal life span greatly exceeded DR24 (Fig. 3a). We did not follow individual animals but an estimate of the relative duration of the adult versus juvenile phases can be calculated by subtracting juvenile duration (Table 2, mean maturation age) from the maximal longevity (Table 1) for either sex. Interestingly, DR had relatively small impacts on maturation age compared to DD, suggesting that DR mainly extended adult longevity (see Fig. 5). The estimated duration of the adult female phase was 1.42 and 1.77-fold longer than DRC for DR24 and DR36, respectively. The latter was the greatest prolongation of the adult phase in any treatment. *Drosophila* females also benefit more from dietary restriction (via DD) than males (Magwere et al. 2004; Bross et al. 2005). Males showed a 1.33-fold increase in adult duration on DR24, but a reduction on DR36 (83% of DRC). In the latter case modest extension of overall male longevity on DR36 (1.18-fold greater than DRC) derived from extension of the juvenile period (clearly evident in Fig. 5).

Extension of longevity by DD can be complicated by compensatory costs (see Metcalfe and Monaghan 2001, 2003). Partridge et al. (2005) found peak longevity occurred at intermediate DD in adult *Drosophila*. Extension of the juvenile period on DD40 may reflect inability to secure sufficient resources to allow maturation as less than half of DD40 males matured (Fig. 5). It remains that DD40 males lived longer than any other group and more than 2 months longer than DDC (i.e., delayed maturation extended longevity far beyond normal limits). The increase in overall lifespan of males on DD40 compared to DDC (1.32-fold) was **entirely** due to delayed maturation (1.65-fold longer than DDC). At 70 days, 78% of crickets on DD40 were male. Moreover, the last 20% of the initial DD40 population outlived DDC and all were males (Fig. 3). Alternatively, adult females expressed reduced lifespan on DD40 (63% of DDC) but overall longevity was unchanged as delayed maturation (1.45-fold longer) balanced earlier adult demise. These trends indicate that DR and DD impacted cricket life cycles and aging differently and these impacts appear to vary between genders. Pearl (1928) cites an example where DR of *Lymantria dispar* caterpillars extended lifespan by 25%–30% entirely by prolonging the caterpillar stage. Delayed maturation and delayed adult senescence appear to be separate mechanisms that can both extend overall life span.

DD40 survivorship resembled DR36 but with even greater life extension (compare Fig. 3a and b). This was associated with high juvenile mortality, however, and further dilution (DD55 and DD75) dramatically foreshortened survivorship. An expert reviewer suggested that juvenile mortality associated with DD may reflect difficulty processing higher-fiber diets. This is possible, but there was greater intake over controls at least for DD25 (Fig. 2).

Growth rates, maturation age and maturation size Body size, growth rate, and time to maturity are critical and inter-related life history features. Resource shortfalls can reduce growth rates, stunt adult size, prolong development and increase risks of mortality and reproductive failure (Scriber and Slansky 1981; Jones and Raubenheimer 2001; Berner et al. 2005). Adjustments in growth rates, size and age at maturity under stress (Table 2) likely reflect adaptive plasticity that ensures reaching reproductive age or that minimizes losses in fecundity or reproductive gains associated with rapid maturity. The relationship between maturation size and age differed strongly with gender on DR versus DD (Fig. 4). Some gender differences may also reflect differential nutritional impacts of a fixed diet.

For males, compensation for mature size may benefit spermatophore production, contest competition and mate choice by females. DR males showed remarkable compensation for mature size, those on DR24 and DR36 obtaining weights 106% and 117% of DRC, respectively (Table 2). This contrasts with DD where male mature size declined progressively with dilution (Table 2). DD likely imposed greater stress on males than DR as indicated by a 61%

reduction in growth rate on DD40 (Table 2). Differences between DR and DD were also evident in relationships of male maturation size to maturation age (compare Fig. 4a, b). DD40 male maturation mass was ~40% lower than DDC as predicted (i.e., actual: 116.5 mg, predicted: 111.2 mg). Thus, compensatory feeding on DD40 (nearly double the intake of DDC) was ineffective or directed elsewhere.

We initiated restriction with juveniles as DR of vertebrates is more effective when initiated in youth. Studies restricting crickets at maturity are underway. DR delays maturation of vertebrates and both DR and DD delayed maturation of crickets (Table 2, Fig. 5). Compensatory feeding failed to maintain intake or growth rates at control levels but size was defended by males on DR via prolonging maturation (Table 2). Maturation age of DR36 and DD40 males was 134% and 165% of respective controls (Table 2, Fig. 5). Oviposition of female *Teleogryllus* crickets peaked in early adulthood whereas males increased calling across their lifespan (Maklakov et al. 2009), suggesting stronger selection for early reproduction in females. *Acheta domesticus* normally engages repeated rounds of oviposition (Woodring et al. 1979) and reproduction generally impacts longevity. We completely prevented oviposition by withholding soil. This may have benefited longevity, although females still produced and carried a complement of eggs (not quantified).

Male *A. domesticus* appear to have lower priority for early reproduction than females, which may allow them to delay maturation to defend mature size and obtain extended longevity via prolonged immaturity (Table 2). Interestingly, male size increased with maturation age on DR, but even greatly delayed maturation could not defend male body size on DD40. Consequently, late maturing males were smaller (Fig. 4a, b).

Life extension with DR was consistent with theory that slower growth (even if prolonged to achieve larger size) can extend longevity (within genders) (Rollo 2002). This was not congruent, however, with both faster growth (Table 2) and greater longevities (Table 1) of females compared to males. DRC males grew at rates only ~55% that of DRC females (Table 2). Growth rates are not simply maximized but represent an optimized life history feature capable of adjustment. Adaptive evolution of growth rates may take into account potential costs (Gotthard et al. 1994; Rollo 2002). Compensation in growth rates was observed, particularly in DR24 and DR36 (Table 2). Both DR36 genders also expressed similar compensation in growth rates (about 87% of respective DRC, Table 2) suggesting that this did not contribute to gender dichotomy in life spans (Table 1).

DR and DD females showed strong compensation for mature size, particularly on DR36 and DD40 (Table 2). Although females on DR24 were ~20% smaller, their growth was reduced by ~30% and feeding by ~50%. Thus, body size reflects compensation at several levels. Growth rates of DD females were generally lower than those on DR (Table 2) suggesting greater stress. Compensatory costs and nutritional stress may have prevented life extension of DD females. Depression of growth rate in DD40 females compared to DDC (70%) was only slightly lower than predicted by dilution. This may explain the higher early mortality of young females and that associated with maturation on DD40. In DD55, compensatory capacity was surpassed leading to early death and lack of maturation. Males may compensate better for DD because of their intrinsic lower growth and maturation mass whereas the intrinsically higher set points for females could derive higher mortality. Rollo and Hawryluk (1988) documented a similar circumstance where high rate r-selected snails compensated less for DD than slower-growing K-selected species. Although some differences between DR and DD likely traces to scheduling and effectiveness of compensatory adjustments, the quality of the control diets also differed. Thus, DD females weighed ~40 mg less than DR females whereas DD males weighed ~35 mg more than DR males.

Conclusion

Advantages of crickets for aging studies include short lives, the ability to include nymphal stages, simple manipulation and scoring of oviposition, a clear biomarker for male reproductive effort (singing) and omnivorous, easily manipulated diets. Intermittent feeding obtained extended longevity in *A. domesticus* comparable to DR in vertebrates, a method that cannot be applied to *Drosophila*. Overall, DD results do not resemble those obtained with *Drosophila*, but we have not yet examined DD restricted to adult

crickets. DD results highlighted delayed maturation as a related but separable mechanism to deferral of adult senescence for life extension. Results generally highlight that compensatory adjustments vary with the method and degree of restriction, and involve a complex interplay of feeding, growth and life-history features (i.e., compensation is multidimensional).

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