

Lifespan extension by caloric restriction is determined by type and level of food reduction and by reproductive mode in *Brachionus manjavacas* (Rotifera)

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ABSTRACT

We measured lifespan and fecundity of three reproductive modes in a clone of the monogonont rotifer *Brachionus manjavacas* subjected to chronic caloric restriction (CCR) over a range of food concentrations or to intermittent fasting (IF). IF increased lifespan 50 – 70% for all three modes, while CCR increased lifespan of asexual females derived from sexually- or asexually-produced eggs, but not that of sexual females. The main effect of CR on both asexual modes was to delay death at young ages, rather than to prevent death at middle ages or to greatly extend maximum lifespan; in contrast CR in sexual females greatly increased the lifespan of a few long-lived individuals. Lifetime fecundity did not decrease with CCR, suggesting a lack of resource allocation trade-off between somatic maintenance and reproduction. Multiple outcomes for a clonal lineage indicate that different responses are established through epigenetic programming, while differences in lifespan allocations suggest that multiple genetic mechanisms mediate lifespan extension.

INTRODUCTION

While caloric restriction (CR) is the only mechanism known to increase lifespan in a wide range of taxa, there is no consensus on the origin of, or mechanisms controlling, this phenomenon (1-3). One hypothesis is that the response to CR evolved to cope with periodic food shortage in the environment, allowing an individual to survive until food again becomes available and reproduction can resume. Implicit in this premise is that there is a trade-off between reproduction and somatic growth and maintenance, and that when resources are limiting energy is not available for both. The presumption of a trade-off is supported by some but not all studies (4-8) and requires further testing. A trade-off between fecundity and lifespan also suggests that different modes of reproduction within the same species (sexual versus asexual, males versus females) might have different responses to CR. It is often difficult to test and distinguish differences in lifespan and fecundity responses to CR due to reproductive mode from those due to interspecific differences.

Caloric restriction extends lifespan in many, but not all, monogonont rotifer species, at least under conditions tested thus far (4, 5, 7, 9). Monogonont rotifers are basal, triploblast, microscopic, aquatic invertebrates with a number of advantages as a model system for the study of aging (10). Their small size and ease of culturing allows testing of multiple treatments with a high degree of replication. As facultatively sexual organisms, monogonont rotifers generally reproduce asexually, with a diploid female producing diploid eggs by mitosis that hatch into other asexual (amictic) females, giving rise to a clonal population. In

response to a quorum sensing mechanism, sexual (mictic) females are produced that generate haploid eggs through meiosis. If unfertilized, these haploid eggs hatch into haploid males. If fertilized, a diapausing diploid egg is formed, which hatches into an amictic female. These diapausing or “resting” eggs may overwinter or be desiccated, and hatch in response to positive environmental conditions. Males do not feed, so studies of CR are conducted on amictic females arising from sexually-derived diapausing eggs, amictic females hatching from mitotically-derived asexual eggs, or mictic females hatching from mitotically-derived asexual eggs.

Lifespan extension in calorically restricted rotifers is sometimes associated with a decline in fecundity, lending support to the idea that reproduction and somatic maintenance are mutually exclusive endeavors under resource limitation. In low nutrient environments, the *Brachionus plicatilis* Ishikawa strain doubled its lifespan but decreased fecundity to less than half that under *ad libitum* (AL) food conditions (9). The mean lifespan and relative fecundity of *Cephalodella* sp. were both 50% lower in CR animals than in AL animals, while *Elosa worallii* had a 50% increase in lifespan offset by a near cessation of reproduction in CR animals (7). Additional studies have shown that starvation prior to first reproduction increased lifespan more than starvation after the onset of reproduction, and that starved rotifers had a smaller body size, suggesting a trade-off between somatic maintenance, longevity, and reproduction (8).

Many of the previous studies on the effect of CR on longevity and reproduction in rotifers have been in the context of the ecology of resource

limitation and population dynamics, although rotifers have also been used to test multiple evolutionary theories of aging (5, 11). Unfortunately, most studies that found a lack of lifespan extension compared distantly related species and examined only one or two levels of CR, so it is unclear whether the optimally restricted diet for each species simply was not tested. These studies generally examined the effects of either differing periods of starvation or chronic caloric restriction (CCR), but not both, for amictic female rotifers of a single or distantly related species (4, 5, 7, 9, 12). This earlier work has provided useful insights about the maintenance of populations in the field or of lifespan under particular food regimens, but a more comprehensive assessment of multiple reproductive modes and a variety of levels and types of CR could provide a better understanding of the origins and mechanisms of CR-induced longevity.

In this study, we measured lifespan and fecundity of three different reproductive modes of the monogonont rotifer *Brachionus manjavacas* subject to six food concentrations ranging from AL feeding to starvation, and to alternate day AL feeding and starvation. By examining a range of CR levels and regimens for multiple reproductive types within a clonal isolate, we were able to dissect the plasticity of lifespan and reproductive responses and relate these to potential origins and mechanisms.

METHOD

Cultures

An isolate of the monogonont rotifer *Brachionus manjavacas* (13), collected from the Azov Sea region in Russia and propagated continuously in the lab since 1983 with periodic resting egg collection and storage, is our model system for aging studies. Rotifers were fed the chlorophyte *Tetraselmis suecica*, which was maintained in 2 L flasks of gently bubbled, 15 ppt artificial seawater (ASW) f/2 medium (Guillard). Both rotifer and algae cultures were kept at 21 °C on a 12:12 h light:dark cycle. Cultures of *T. suecica* used for CR studies were maintained in semi-continuous log phase growth by daily removal of approximately 20% of the culture and replacement with f/2 medium. Maternal females were maintained in *ad libitum* (AL) food conditions for at least one week prior to experiments to prevent known lifespan-extending maternal effects of CR on offspring (14).

Experimental Conditions

In this study, we conducted life table experiments to examine the effects of seven different food concentrations on lifespan and reproduction for three reproductive modes of *B. manjavacas*. The diapausing, or “resting,” eggs of *B. manjavacas*, the result of sexual reproduction, were collected from 200 L batch cultures, desiccated, and stored at -20 °C for approximately two years before these experiments. Amictic eggs were removed from mature amictic females by vortexing and then isolated by micropipette. Neonates of both diapausing and

amictically derived eggs < 3 h old were individually isolated into 1 ml of *T. suecica* at a concentration of 6×10^5 cells ml⁻¹ (equal to 100% of AL) in 24-well plates. The AL concentration was chosen based on previous aquaculture studies that showed maximal grazing and specific growth rates in *Brachionus* rotifers under comparable food conditions (15-17). All individuals were fed at AL concentrations for the first 20 h then maintained individually in 1 ml at the treatment food concentrations for the duration of lifespan. Caloric restriction was attained by diluting algae with 15 ppt ASW to 75%, 50%, 25%, 10% and 0% of AL concentrations. Intermittently fasted (IF) individuals were fed on alternate days at 100% and 0% of AL.

Each day of the experiment, lifespan, reproductive status (pre-reproductive, reproductive, or post-reproductive), and number of offspring were scored, and the original female was moved to a new well with clean water and *T. suecica*. Amictic females hatched from sexually-derived resting eggs (RE-amictic) and amictic females hatched from mitotically-derived amictic eggs (AE-amictic) both produce only amictic, diploid female offspring. Mictic females hatched from mitotically-derived amictic eggs (AE-mictic) produced haploid eggs by meiosis; as the AE-mictic females did not copulate prior to egg production their eggs developed as haploid males. Kaplan-Meier survival curves and tests for significance were calculated using Prism 5.0d and Excel 2008. Daily age-specific hazard rates (μ_x) were determined using the simplified version of the Sacher estimate: $\mu_x = -\ln(1-q_x)$ where q_x is the age-specific probability of death in

the interval Δx (18). Hazard rates were calculated for each treatment until only four individuals persisted.

RESULTS

Effect of Caloric Restriction on Lifespan

The three reproductive types of *B. manjavacas* had different trends in survival curves across the range of food concentrations (Figure 1). Survival curves for amictic females hatched from resting eggs (RE-amictic) had gradual slopes, while those for amictic or mictic females hatched from amictic females (AE-amictic and AE-mictic) were in general steeper with a flattening of the tail caused by the extended survival of only a few individuals over the last days of the experiment. For amictic females under CCR, a significant extension of lifespan was not seen until food was restricted to 50% of AL or less. Survival curves, hazard rate curves, and mean and median lifespan measures indicated that there was no change in lifespan until food concentration dropped below a threshold of 3×10^5 cells ml^{-1} (Figures 1-3, Table 1). Below 50% of AL feeding, decreasing food concentration led to increasing mean, median, and maximum lifespan, but the magnitude of the increase depended upon reproductive mode.

For RE-amictic females, 50% and 10% of AL produced nearly identical survival curves and a 50% increase in mean and median lifespan of over those for AL feeding. A maximum lifespan of 28 days was reached under 10% of AL. AE-amictic females had gradually increasing median lifespan from eight days at 100% of AL to 10 days at 10% of AL. AE-mictic females, on the other hand,

exhibited significantly extended lifespan only under IF. Uniformly across reproductive types, individuals in the starved treatment (0% AL) began dying on day four, and all were dead by day seven.

The greatest extension in mean and median lifespan occurred for all reproductive types in IF individuals, with an increase in mean and median lifespan of 150 – 171% that of individuals fed AL (Figures 1, 3; Table 1). Maximum lifespan increased under IF, but was greater than 10% CCR only in AE-mictic females. Restriction of food to 10% of AL levels produced the next highest increase in mean and median lifespan in all reproductive types.

Hazard rate curves (Figure 2) indicated that the main effect of both types of CR on amictic females was to delay death at young ages, rather than to prevent death at middle ages or to greatly extend maximum lifespan, a phenomenon also reflected in the survival curves. In AE-mictic females, CR did not decrease the hazard rate at early or mid-ages, but 10% CCR and IF greatly increased the lifespan of a few long-lived individuals.

Effect of Caloric Restriction on Lifespan Allocation and Fitness

Caloric restriction altered the allocation of lifespan to pre-reproductive, reproductive, and post-reproductive periods differently in amictic and mictic females (Figure 4). Both types of amictic females significantly increased the reproductive portion of lifespan under IF (to 73 and 84%), while mictic females maintained a relatively constant proportion of lifespan in reproduction across varying levels of CCR and IF (from 51 – 58%). Conversely, mictic females

increased the reproductive portion of lifespan to nearly 72% while starved, while amictic females maintained or slightly decreased their reproductive period under starvation conditions. For both AE-mictic and AE-amictic females, a significantly shorter portion of lifespan was spent in the pre-reproductive stage at very low food concentrations.

No trade-off between lifetime fecundity and lifespan was apparent with declining food levels, except in IF AE-amictic females (Figure 5). In fact, for RE-amictic and AE-mictic females, average lifetime fecundity generally increased with increasing CR. Lifetime fecundity was relatively constant under CCR from 10% to 100% of AL for mictic females. Caloric restriction influenced average daily reproduction over the course of the experiment, however (Figure 6). For amictic females, average daily reproduction was greater at higher food concentrations for the first 5 – 6 days of the experiment, then shifted as females aged to become higher for IF and the lowest levels of CCR. Mictic females had similar daily reproduction across CCR food concentrations, except for IF and starved treatments, where daily reproduction was greatly reduced. Complete starvation led to declines in both daily and lifetime average reproduction in all female types.

Daily reproduction was negatively correlated with lifespan early in life, but positively correlated with lifespan late in life (Figure 7). Negative correlations for amictic females on day three of the experiment were driven by the higher reproductive output of less-restricted females (with shorter lifespan) relative to females subject to extreme CCR or IF. In mid-life, around days 5 and 6, the

association between daily fecundity and lifespan shifted. In late life, the reproduction of less-restricted females (100% and 75% AL) dropped to near zero, while the daily reproduction of longer-lived 10% AL and IF amictic females was maintained. The correlation between daily fecundity and lifespan for mictic females was driven almost entirely by the single point for IF treatment at all ages, since daily reproduction was unchanged by CCR for mictic females.

DISCUSSION

In this study, we surveyed the effects of seven different CR regimens on the lifespan and fecundity of three reproductive modes of the monogonont rotifer *Branchionus manjavacas*. We found that the magnitude of lifespan extension and the concomitant effect on reproduction depended both on the type of CR and on the female's reproductive type, suggesting a diversity of underlying mechanisms for the response to CR.

Threshold Response to Chronic Caloric Restriction

Branchionus manjavacas appears to have a threshold below which the food concentration must drop before eliciting a lifespan-extending response. We did not observe a significant increase in lifespan of RE-amictic females until food limitation reached 50% of AL or less, and further limiting food between 50% and 10% of AL resulted in longer mean and maximum lifespan. Restriction to 25% of AL was required before lifespan increased in AE-amictic females. Previous

grazing and population growth experiments in similar species of *Brachionus* show that maximal specific growth rates are achieved at food levels comparable to our AL concentration and decline with decreasing food concentrations (15-17). We did not measure assimilation rates at the varying food concentrations, however, and at higher concentrations a larger proportion of food may have passed through the rotifer gut without being fully digested or incorporated.

These results suggest induction of the CR response was controlled by nutrient sensing or metabolic status, which was not triggered until perceived or consumed resources reached a critical level. This is consistent with the wide range of rapidly changing resource conditions in the natural habitats of *B. manjavacas*, and implies that different *Brachionus* isolates may have different response thresholds depending on their native environment. On a practical note, these results demonstrate that one must use caution in interpreting studies in which a single level of CR has been tested; as in this study what may appear as a lack of response to CR at 75% of AL, for example, may simply be the incorrect level to induce lifespan-extending mechanisms. Without examination of a range of food levels, it is impossible to determine the level of restriction at which there is maximum longevity with the fewest detrimental side effects.

Varied Response to CR of Different Reproductive Modes

Differences in the response to CR depended not only on the method of food restriction but also on the reproductive mode of the female, likely due to the differences in evolutionary pressures on different types of females. Mictic

females were unable to respond to any level of CCR, but increased mean lifespan by 70% under IF. A similar lack of response to CCR was found previously for mictic females of the freshwater species *Brachionus calyciflorus* (19). The production of mictic females by *B. manjavacas* is induced by environmental conditions primarily driven by crowding, and requires relatively low ammonia conditions (i.e. good water quality) and high food concentrations (20, 21). Mictic females are programmed for maximum reproductive output as quickly as possible, to allow sexual reproduction and the production of diapausing eggs that can overwinter and withstand desiccation and thereby permit the population to persevere through adverse environmental circumstances. A mechanism to delay reproduction and extend lifespan in low food conditions thus would not benefit mictic females or ensure passage of their genetic material, since a declining population caused by delayed reproduction would not allow the frequent random encounter between males and mictic females that is required for mating. In addition, there is a waning ability of males to recognize and mate with mictic females more than 24 h. old, further decreasing the evolutionary incentive of mictic females to live to old age (22).

Amictic females, on the other hand, had increased lifespan for both CCR and IF, but with different reproductive strategies under each. Under IF, average daily reproduction was lowered almost immediately and the reproductive period was extended in proportion with the extended lifespan, to result in the same lifetime fecundity. Amictic females would benefit from an ability to survive through low resource conditions, as their “best bet” for evolutionary success is to

increase the chances for survival of their offspring by producing eggs in a hospitable environment.

Previous studies have shown that RE-amictic females are unable to respond to mixis cues, and only a portion of AE-amictic female population can produce mictic females, although all are exposed to the same cue and all are genetically identical (20). This strong evidence of “bet-hedging” by maintaining multiple reproductive modes within the same population is also likely to influence lifespan responses to CR.

Differences Between Chronic Caloric Restriction and Intermittent Fasting

Differences between daily fecundity and allocation of lifespan to reproduction under CCR versus IF suggest that different mechanisms are employed to promote longevity under the different types of food limitation. Mean lifespan was greatest under IF for all reproductive types, but amictic females responded to both CCR and IF with extended lifespan, while mictic females had increased lifespan only under IF. Additionally, amictic females greatly increased the reproductive portion of lifespan under IF, but not under CCR, while mictic females maintained a constant reproductive portion of lifespan under all types of CR except starvation.

Studies in many other animal systems have reported differences in the effects of CCR and intermittent caloric restriction (ICR) or IF. Reports in multiple species, from *C. elegans* to mice, have verified that IF or ICR frequently increase longevity more than does CCR (23). Additionally, IF/ICR and CCR have diverse

effects on other age-related phenotypes. Mice subjected to ICR have lower levels of mammary tumor formation, with corresponding lower IGF-1 and mTOR levels, than do mice under CCR or AL, for example (24).

Despite noticeable differences in IF/ICR and CCR phenotypes in many animal systems, the possible differences in mechanism are unclear. Few studies have addressed the genetics of multiple CR regimens simultaneously, even though cross-study comparisons are problematic, and direct comparisons could help tease apart mechanisms (25). In *C. elegans*, diverse CCR regimens and CR mimetics evoked a variety of independent genetic pathways involved in nutrient sensing and stress response, though IF was not tested (26). Studies of IF in which animals had same overall caloric intake as AL animals, but still exhibited increased lifespan and stress-resistance, argued for an indirect effect of energy sensing on anti-aging response in IF regimens (25).

Brachionus rotifers in their natural habitats are unlikely to see food conditions like those of the IF treatment imposed in this study. In the wild, *B. manjavacas* would consume a variety of microbial species, and phytoplankton and bacterial dynamics in the rotifers' aquatic habitats are much more likely to demonstrate long periods of very low or high concentrations, gradually increasing or decreasing food concentrations, or complete depletion for a period of time, rather than a simple every-other-day AL and starvation conditions. Thus, it seems probable that while a mechanism for adjusting lifespan and reproduction would have evolved in response to CCR, IF may simply be invoking a stress response in *B. manjavacas*.

The results of our study highlight the value of examining an array of phenotypes when studying the effects of CR. Many reports of lifespan-extending interventions in invertebrates employ fluorodeoxyuridine (FUdR), an inhibitor of DNA synthesis, to prevent hatching of offspring, making tracking of individual animals much easier. In this study, however, because maximum and medium lifespan were similar for 10% and IF, we would not have seen that lifespan extension was occurring by different processes in the two types of CR without also measuring reproduction. Further, the lifespan altering properties of FUdR itself are poorly understood even for eutelic organisms, and are rarely tested against a non-FUdR control in invertebrate aging studies. Additional parameters beyond lifespan should be measured in studies of CR, to shed light on potential differences in mechanisms with varied modes of food limitation.

Resource Allocation Under Caloric Restriction

Analysis of a potential trade off between fecundity and lifespan under limiting resources revealed a complex system in which initial average daily reproduction was lowered, but late-life daily reproduction and lifetime fecundity were increased, relative to AL. If there were a straightforward trade-off between lifespan and reproduction, one might expect continually decreasing daily reproduction, and a resulting lower lifetime reproductive output, to be associated with increasing lifespan in more restricted individuals. Instead, in both mictic and amictic females hatched from amictic eggs, the pre-reproductive period shortens with both CCR and IF. This suggests a strategy to initially produce offspring as

quickly as possible under limiting conditions, an observation that has been made previously in the monogonont *Synchaeta pectinata* (12). Over the course of our experiment, however, we observed a shift in daily reproduction between AL and CR rotifers: daily reproductive output was initially higher in AL-fed rotifers, but then switched in late life so that higher daily reproductive rates were maintained in CR individuals but declined in AL fed rotifers. In fact, there was a flat or negative correlation between lifespan and mean daily fecundity during the period of maximal reproduction on days 2-5, but in later life lifespan and mean daily fecundity were positively correlated in all reproductive types. Resource allocation trade-offs thus may be confined to the early reproductive period, as was also reflected in the decreased hazard rate for CCR and IF rotifers in early but not in late life. Unseen trade-offs may have manifested as decreased body size, offspring viability, or long-term specific growth rate, parameters not measured in our experiment.

The evidence for a trade-off between reproduction and lifespan in the face of food shortage is mixed. One of the primary arguments in favor of a trade-off is that when rotifers are starved later in life, after beginning reproduction, they have lower tolerance to starvation than rotifers starved early in life (4). Additionally, many rotifer species immediately cease reproduction upon starvation, and resume reproduction upon reintroduction of feeding (7). In some studies in which rotifers are starved, either for different lengths of time or at different times in their lifespan, lifetime fecundity is indeed decreased, much as we observed for rotifers starved after the first day of the experiment (7). However, there are many reports

where reproduction is decreased but lifespan is not extended, particularly when starvation occurs late in life or for extended periods (5, 7, 8, 12). Moreover, it has been demonstrated that even without food restriction, reproduction near the end of life is more closely associated with a short lifespan than is early reproduction (6). In addition to our observations of increasing lifetime reproduction with decreasing food concentrations, others found that *S. peicinata* increased the proportion of energy allocated to reproduction as food became more limiting, producing a constant egg size but reproducing earlier under limiting conditions, and that there was no significant difference in lifespan between restricted and unrestricted rotifers (12).

Unfortunately, evidence on both sides is predominantly correlative; it is experimentally difficult to establish a causative relationship between energy intake, reproduction and lifespan. Our findings support the hypothesis that amictic monogonont rotifers have a temporally dynamic reproductive strategy, as has been found for organisms ranging from plants to rodents to birds, which allows them to deal with food limitation and to resume reproduction when conditions are favorable, and which may or may not be directly linked to lifespan extension (8, 27). Such a strategy may become less flexible with age, for reasons unrelated to food concentrations. The age-specific physiological constraints due to changes in molecular pathways and the accumulation of DNA and cellular damage with increasing age provide as plausible a causative force in decreased starvation tolerance with age as does the allocation of limited resources to reproduction (28).

CONCLUSIONS

Examination of lifespan and fecundity in *B. manjavacas* subjected to food limitation leads us to hypothesize that different mechanisms are employed under different CR regimens. Additionally, studying multiple reproductive modes within a clonal isolate of a single species enabled us to dissect the plasticity of lifespan and reproductive responses in the face of potentially conflicting evolutionary programs, without the confusion of multiple genotypes. We hypothesize that in amictic females a single mechanism invoked by a threshold response promotes increased longevity under CCR conditions, while different pathways, possibly related to stress response, are evoked by IF. The main effect of both types of CR was to delay death at young ages, rather than to prevent death at middle ages or to greatly extend maximum lifespan. In mictic females there was no increase in longevity from any level of CCR; the positive response to IF may be due to yet a third mechanism. This contrast between genetically identical organisms suggests different genetic responses are established through epigenetic programming early in development, probably due to the different reproductive strategies of asexual and sexual females. Based on studies in other organisms, CCR-induced longevity increases maybe due directly to changes in energy intake, while IF-induced lifespan extension maybe an indirect effect of differences in resource intake, leading to upregulation of stress response mechanisms (25). Further dissection of genetic mechanisms will determine if IF and CCR work via a single universal process across reproductive types, or by

overlapping or distinct nutrient sensing, metabolic, and/or hormetic pathways in rotifers and other animals.

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FIGURE LEGENDS

Figure 1. Kaplan-Meier survival curves for (A) RE-amictic (B) AE-amictic and (C) AE-mictic females of *Brachionus manjavacas* subjected to constant caloric restriction at 0 – 100% of *ad libitum* feeding, or to intermittent fasting (IF). * indicates significant difference between treatment and 100% *ad libitum* feeding ($p < 0.05$, Mantel-Cox test).

Figure 2. Age-specific hazard rates for (A) RE-amictic (B) AE-amictic and (C) AE-mictic females of *Brachionus manjavacas* subjected to constant caloric restriction at 0 – 100% of *ad libitum* feeding, or to intermittent fasting (IF). Hazard rates were computed when four or more individuals were present, as samples sizes late in life were too small to adequately assess the hazard rate (18).

Figure 3. Mean lifespan for RE-amictic, AE-amictic, and AE-mictic females of *Brachionus manjavacas* subjected to constant caloric restriction at 0 – 100% of *ad libitum* feeding, or to intermittent fasting (IF). * indicates significant difference between treatment and 100% *ad libitum* feeding ($p < 0.05$, two-tailed t-test with Bonferroni's correction).

Figure 4. Proportion of lifespan allocated to pre-reproductive, reproductive, and post-reproductive stages in (A) RE-amictic (B) AE-amictic and (C) AE-mictic females of *Brachionus manjavacas* subjected to constant caloric restriction at 0 – 100% of *ad libitum* feeding, or to intermittent fasting (IF). * indicates difference in that stage from comparable stage in 100% *ad libitum* feeding treatment ($p < 0.05$, two-tailed t-test with Bonferroni's correction).

Figure 5. Mean lifetime individual fecundity of RE-amictic, AE-amictic, and AE-mictic females of *Brachionus manjavacas* subjected to constant caloric restriction at 0 – 100% of *ad libitum* feeding, or to intermittent fasting (IF). * indicates significant difference between treatment and 100% *ad libitum* feeding ($p < 0.05$, two-tailed t-test with Bonferroni's correction).

Figure 6. Mean daily individual fecundity of (A) RE-amictic (B) AE-amictic and (C) AE-mictic females of *Brachionus manjavacas* subjected to constant caloric restriction at 0 – 100% of *ad libitum* feeding, or to intermittent fasting (IF).

Figure 7. Correlation between average daily reproduction and lifespan for RE-amictic, AE-amictic, and AE-mictic females of *Brachionus manjavacas* on (A) day 2, (B) day 3, (C) day 6, and (D) day 8 of the experiment. r^2 value given for linear correlation; starved rotifers (0% AL) were not included.

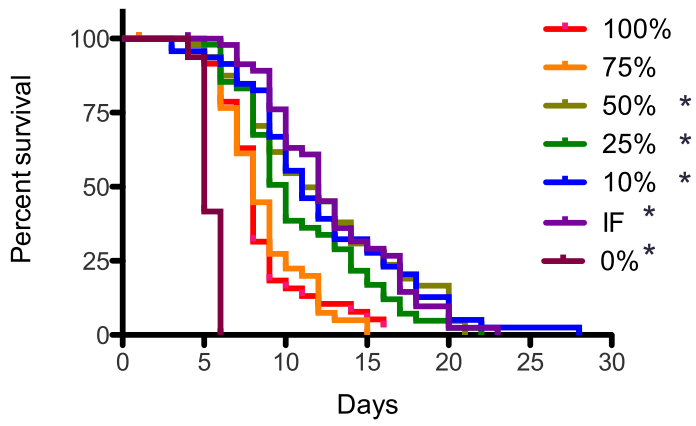
Table 1

	Food		Minimum	Mean	Median	Maximum	Mean Total
	Concentration		Lifespan	Lifespan	Lifespan	Lifespan	Offspring
	(% <i>ad libitum</i>)	n	(d)	(d; SEM)	(d)	(d)	(d; SEM)
	100	41	4	8.1 (0.4)	8	16	24.8 (1.2)
	75	43	5	8.5 (0.4)	8	15	23.2 (1.5)
				12.0			
RE-	50	44	4	(0.8)**	11	21	26.7 (1.7)
Amictic	25	43	5	10.8 (0.6)**	9	22	25.5 (1.8)
	10	43	3	12.1 (0.8)**	11	28	29.2 (2.0)
	IF	44	6	12.7 (0.6)**	12	23	31.1 (1.7)*
	0	48	4	5.4 (0.09)**	5	6	8.0 (0.2)**
	100	49	5	8.7 (0.2)	8	14	31.2 (0.4)
AE-	75	47	4	8.5 (0.3)	8	15	30.9 (0.5)
Amictic	50	43	4	9.2 (0.4)	9	17	31.1 (0.6)

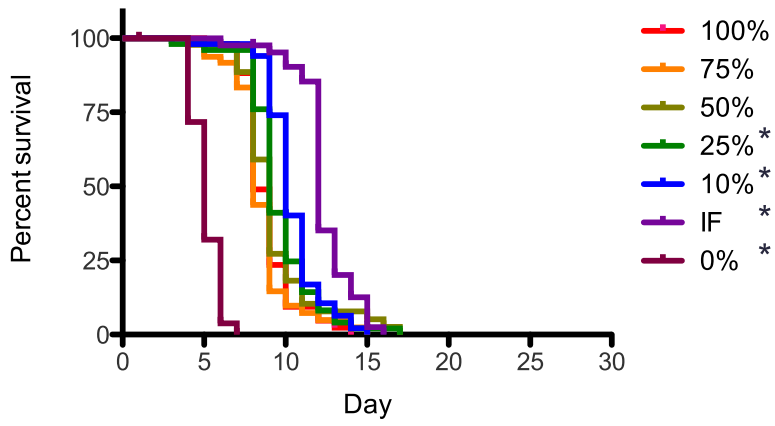
	25	51	3	9.6 (0.3)*	9	17	30.4 (1.0)
	10	48	4	10.3 (0.3)**	10	15	31.1 (0.6)
	IF	40	6	12.3 (0.3)**	12	16	22.2 (0.6)**
	0	53	4	5.1 (0.1)**	5	7	3.8 (0.1)**
	100	20	5	7.5 (0.3)	7	11	29.2 (0.5)
	75	23	5	7.3 (0.3)	7	11	28.8 (0.5)
AE-	50	25	5	7.5 (0.3)	7	12	30.4 (0.3)*
Mictic	25	19	3	7.5 (0.3)	8	9	29.9 (0.9)
	10	21	6	9.0 (0.8)	8	20	31.0 (0.6)*
	IF	30	8	12.5 (0.6)**	12	22	31.6 (0.5)**
	0	18	4	5.1 (0.2)**	5	7	8.5 (0.5)**

n, number of individuals; d, days; SEM, standard error of the mean. The difference between 100% feeding and CR regime for mean lifespan and mean total offspring were tested using two-tailed t-test with Welch's correction for unequal variance (* $p < 0.05$; ** $p < 0.005$)

A) RE-Amictic



B) AE-Amictic



C) AE-Mictic

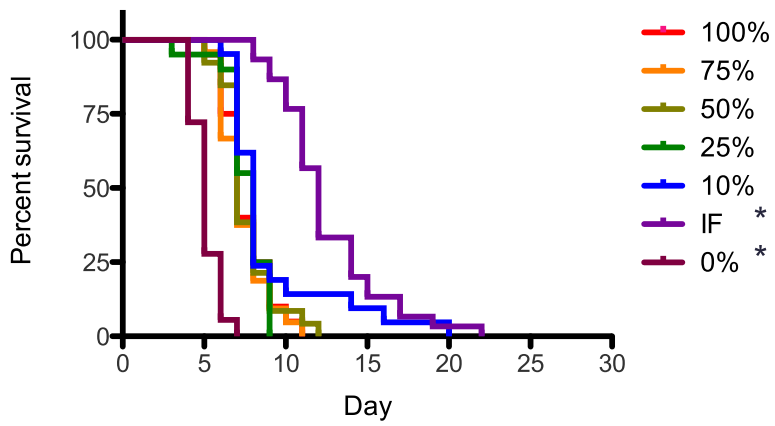
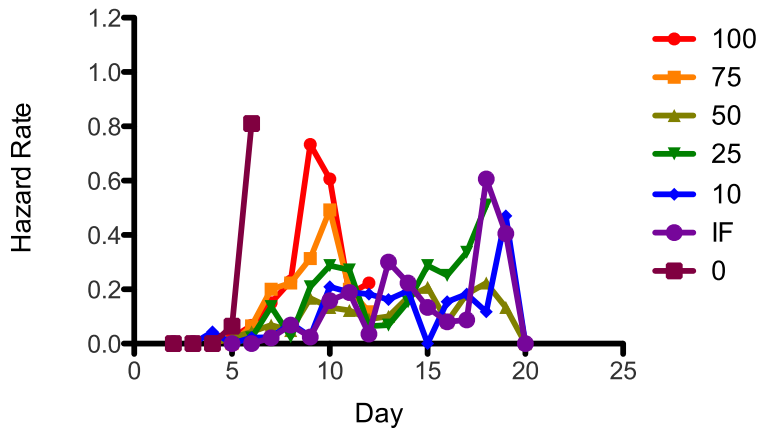
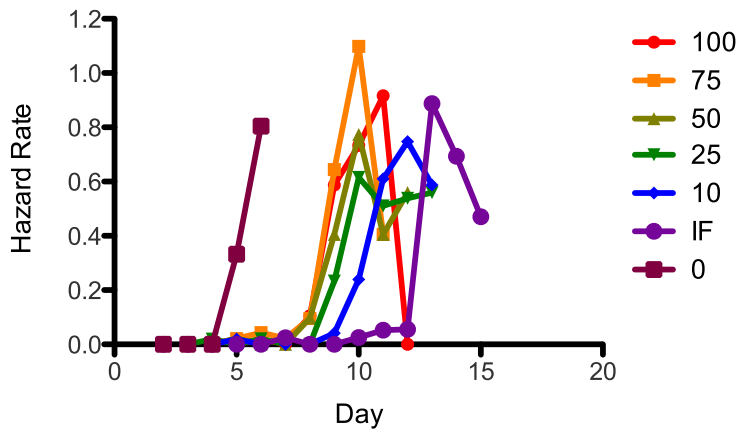


Figure 1

A) RE-Amictic



B) AE-Amictic



C) AE-Mictic

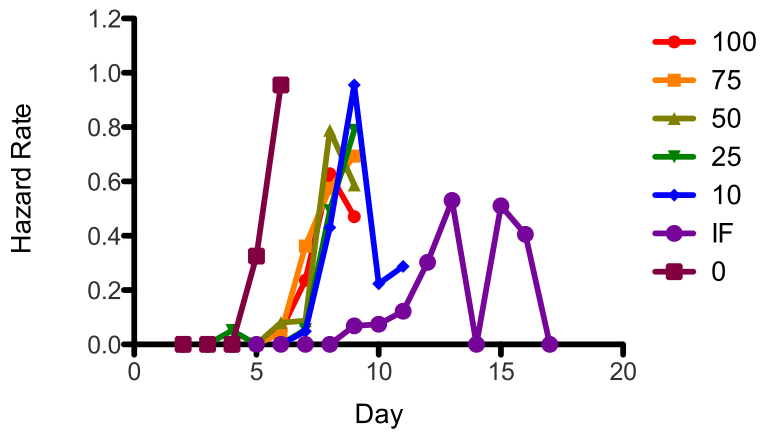
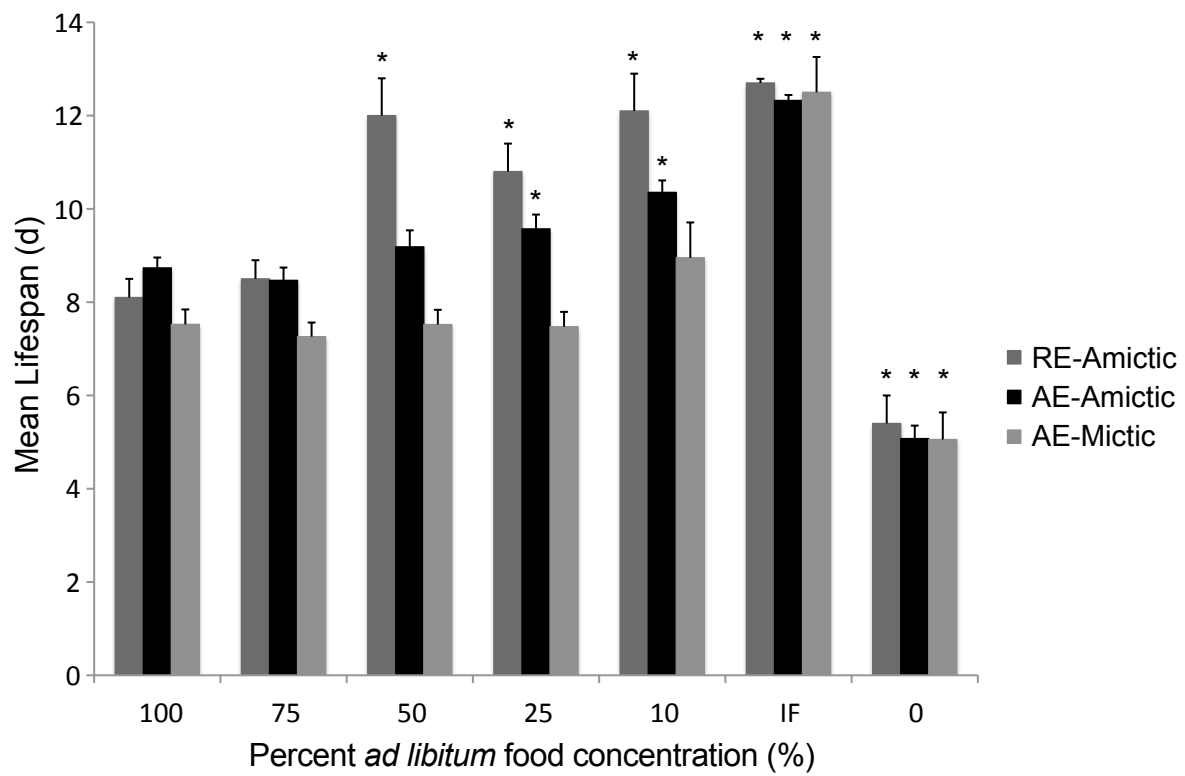


Figure 2



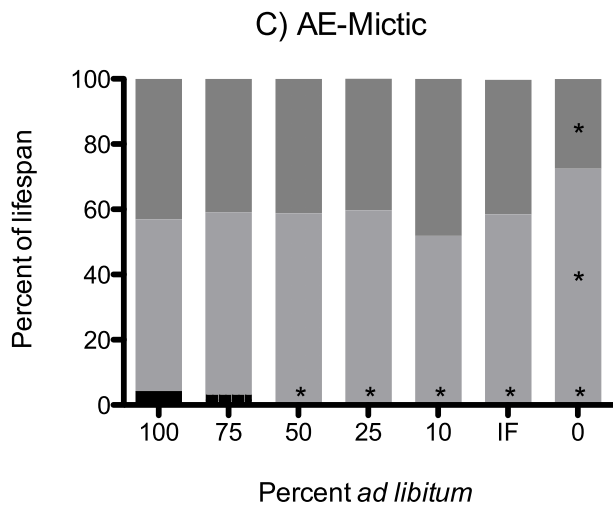
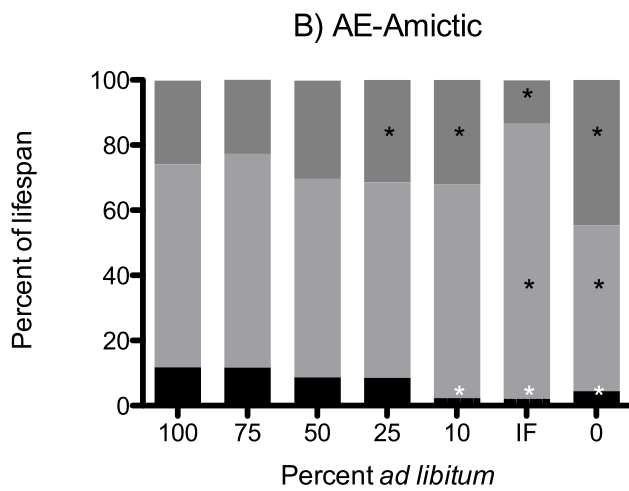
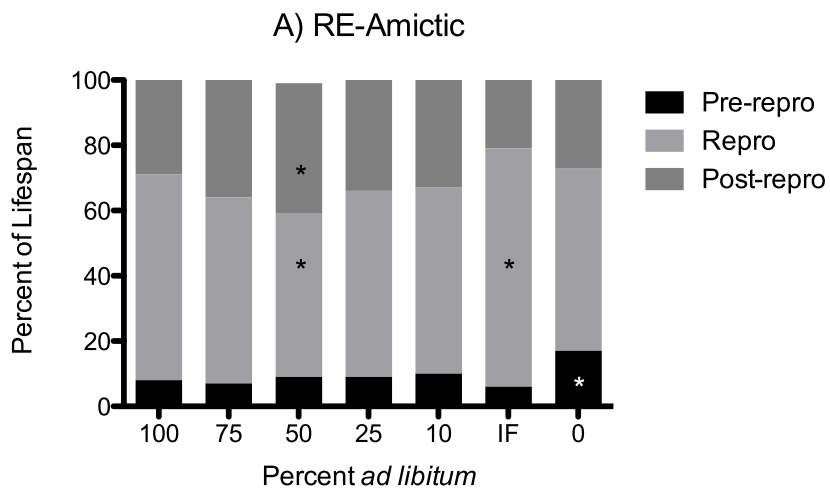


Figure 4

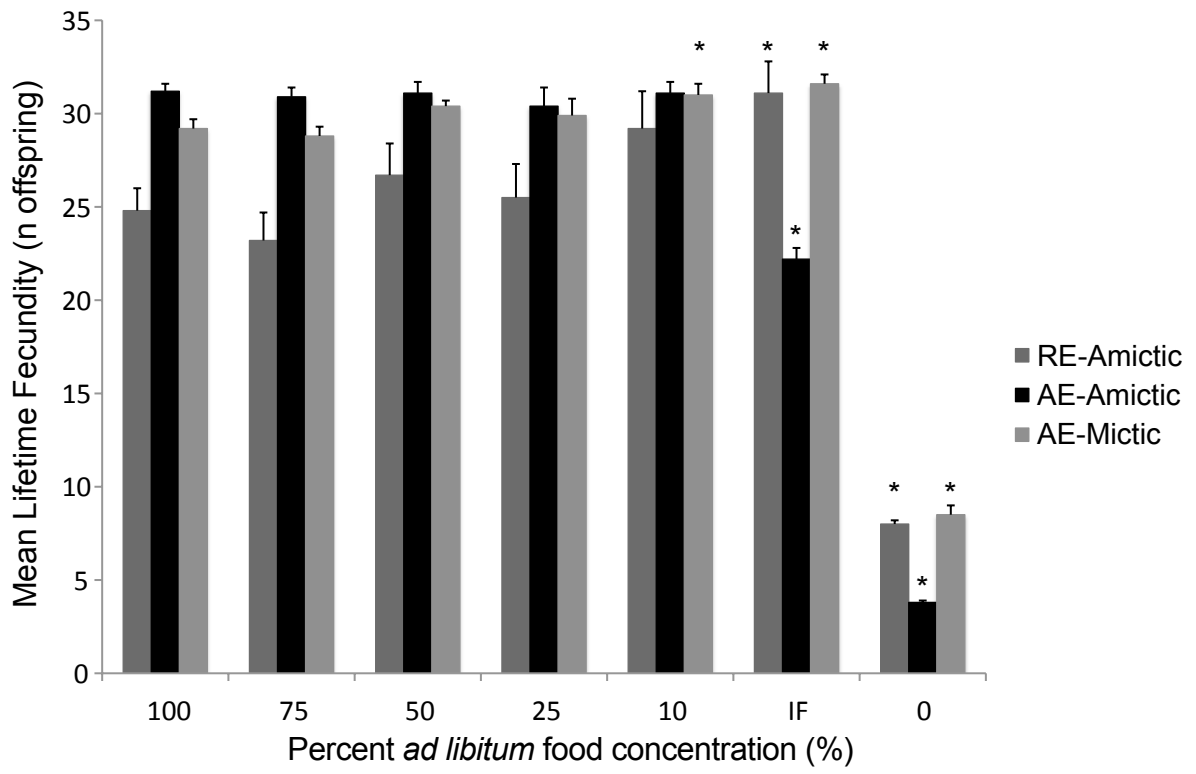
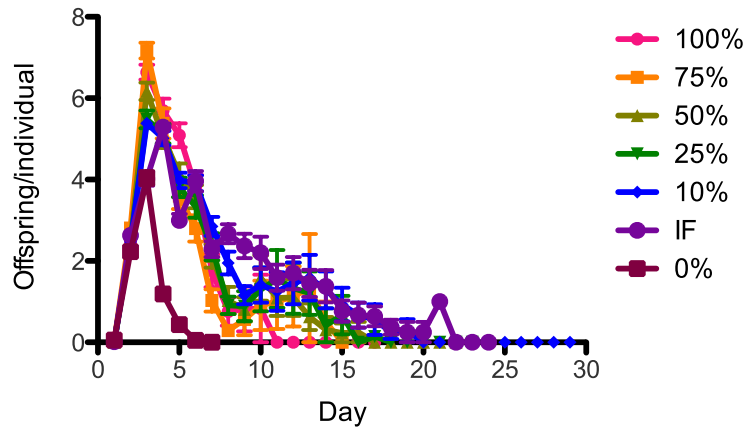
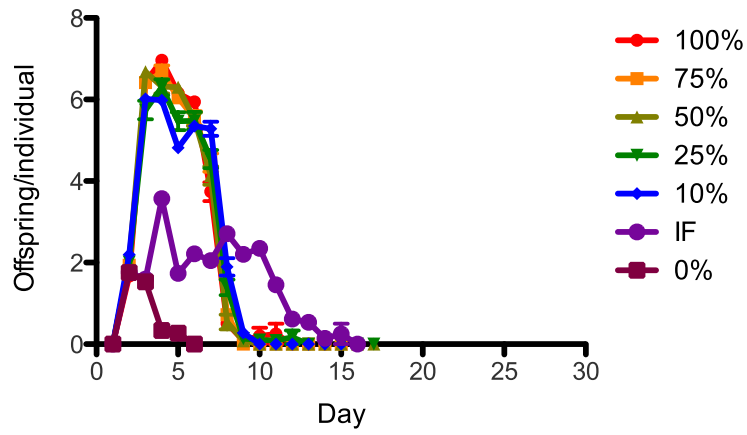


Figure 5

A) RE-Amictic



B) AE-Amictic



C) AE-Mictic

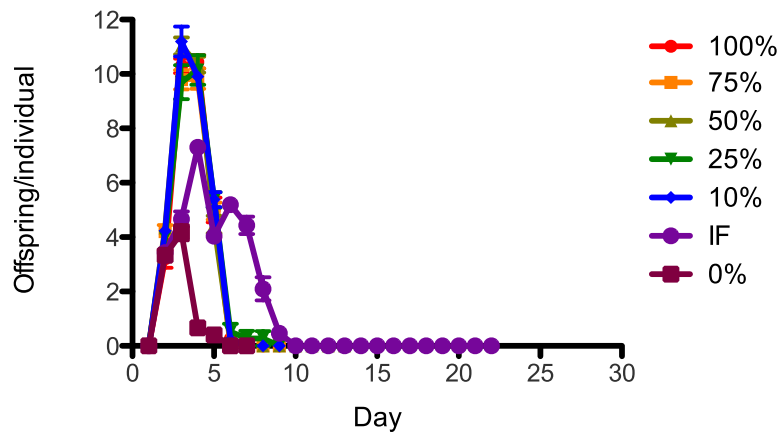


Figure 6

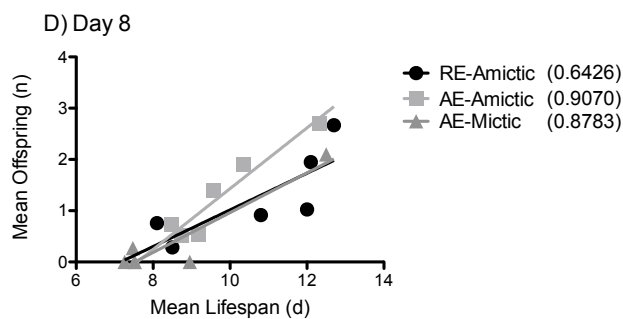
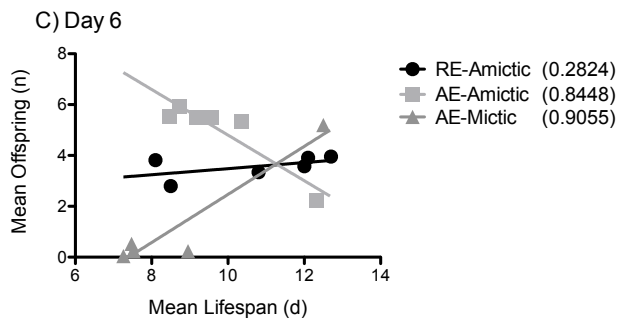
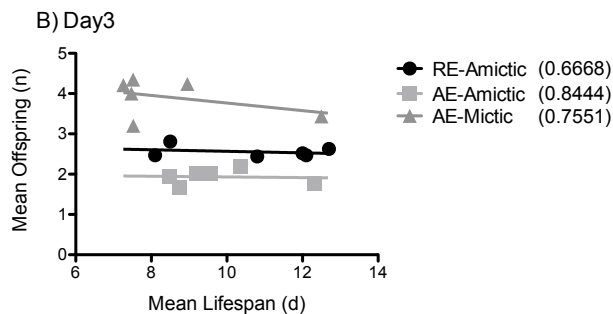
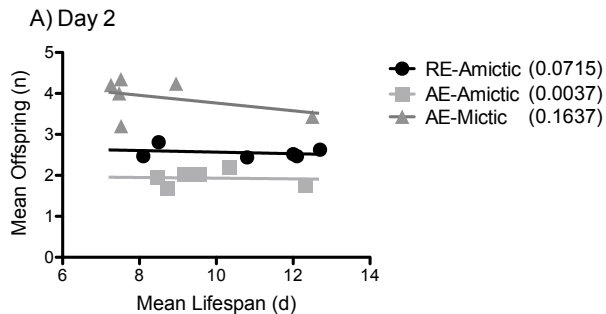


Figure 7