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Life-extending ovariectomy in grasshoppers increases somatic storage, but dietary restriction with an equivalent feeding rate does not

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Abstract

Reduced diet or reduced reproduction each extends lifespan in many animals. It is often thought that reduced reproduction and reduced diet may act through the same mechanisms. In grasshoppers, ovariectomy extends lifespan and reduces feeding to a level similar to that used for life extension by dietary restriction, further suggesting mechanistic overlap. Here, we measure the feeding rate of ovariectomized grasshoppers and, by manipulating feeding levels, create a sham-operated & dietary restricted group with matched daily feeding. Both groups show ~25% increased survivorship near the median age of mortality for fully fed and reproductive controls. Ovariectomy results in a doubling of fat body mass and hemolymph volume in comparison to both a feeding-matched dietary restriction group and a sham-operated & fully fed control, which do not differ from each other. Total anti-oxidant activity in the hemolymph and the skeletal muscle was unchanged upon ovariectomy or dietary restriction, so it does not appear to be a major factor in lifespan extension. Next, we measured mitochondrial counts using qPCR to determine mitochondrial cytochrome-b concentrations relative to nuclear (genomic) beta-actin. Mitochondrial counts in the ovariectomized group were lower than sham-operated and fully fed controls but not than the dietary restriction group. Last, in the fat body, transcript levels of hexamerin-90 (a hemolymph storage protein) were affected by neither ovariectomy nor dietary restriction. Hence, ovariectomy resulted in large magnitude increases in organismal storage. The matched-fed dietary restricted group differed from the ovariectomized group only in organismal storage, and not in any of the cellular parameters measured here. This study suggests that longevity via ovariectomy has distinct physiological mechanisms from longevity via dietary restriction in grasshoppers that are independent of daily feeding rate, particularly for protein and fat storage.

Keywords

reduced reproduction; feeding rate; fat storage; mitochondria

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Introduction

Reduced diet or reduced reproduction each extends lifespan in many animals (Flatt 2011; Nakagawa et al. 2012). Reductions in feeding that are sufficient to extend lifespan typically reduce fecundity as well (Partridge et al. 2005). Thus it is often suggested that reduced reproduction and reduced diet may extend longevity through the same mechanisms, or at least that nutrition influences how reproduction affects longevity (Crawford et al. 2007). However, recent work has begun to identify molecular mechanisms of life extension via reduced reproduction, some of which may be distinct from the mechanisms of dietary restriction (Hansen et al. 2013). For example, germline ablation in *C. elegans* results in activation of pathways of fatty acid desaturation, including the nuclear receptor NHR-80 (Goudeau et al. 2011; similar results in McCormick et al. 2012). Similarly, in a separate study, the transcription factor *tcer-1* was shown to be increased upon germline ablation (Ghazi et al. 2009). Both of these genes are required for longevity via reduced reproduction, but to our knowledge have not been shown to be important for dietary restriction. Yet, the question of whether reduced reproduction extends lifespan by means that are distinct from those of dietary restriction cannot be fully addressed without properly controlling for potential effects of ingestion (Carvalho et al. 2005), and very few studies have measured feeding rates in animals with a direct (i.e., non-dietary) life-extending reduction in reproduction. Indeed, the importance of feeding rate is shown by the ability of dietary restriction to slow tumor growth in mice; this requires Neuropeptide Y, which regulates feeding rate (Minor et al. 2011). These same authors predict that Neuropeptide Y will play a role in life-extension (Minor et al. 2009). Here, we used a matched-feeding approach to test whether several physiological parameters often associated with longevity differ between reduced reproduction and reduced diet.

Grasshoppers (viz., lubber grasshoppers, *Romalea microptera*) are excellent models for studying the physiology of aging in general, and for a comparison of the effects of dietary restriction and reduced reproduction in particular. These animals are sufficiently large so that feeding rate can be easily measured, feeding amount (as opposed to diet quality on an *ad libitum* diet) can be manipulated, and multiple tissues from a single individual can be tested for biochemical parameters (e.g., Fronstin et al. 2008; Judd et al. 2011). This species of grasshopper has potent chemical defenses (e.g., Hatle et al. 2002), so they likely have evolved to be relatively long lived, making experimental extensions of the lifespan more remarkable. In particular, the population used here, sampled from Miami, FL, USA, reproduces later (Hatle et al. 2002) and may be more long-lived than other populations (Gunawardene et al. 2004).

Dietary restriction (i.e., 60% of that eaten by *ad libitum* fed controls) and late-onset dietary restriction (started after first oviposition) both extend lifespan in grasshoppers. Interestingly, the hemolymph levels of protein, a major amino acid pool for egg protein production, are not reduced upon life-extending dietary restriction (Hatle et al. 2006b).

Reduced reproduction via ovariectomy also extends lifespan in grasshoppers, and it results in a ~40% reduction in feeding rate starting at about 40 d, the age at which intact females lay their first clutch (Drewry et al. 2011). This feeding regime is remarkably similar to the late-onset dietary restriction that extends lifespan (Hatle et al. 2006b). Yet, hemolymph protein levels are higher in ovariectomized females than in fully reproductive controls (Hatle et al. 2008).

Life-extension from ovariectomy occurs without blocking allocation of protein to reproduction because the precursor to egg yolk protein (vitellogenin) is still produced. Because the ovary is missing, the vitellogenin accumulates at high levels in the hemolymph

(Hatle et al. 2003, 2008). Further, ovariectomy appears to affect energy balance by increasing fat depots (Judd et al. 2011). While increased fat stores are known to increase upon reduced reproduction in many other animals (Hansen et al. 2013), we have shown in grasshoppers that this hypertrophy occurs despite reduced feeding. Last but not least, longevity via dietary restriction and ovariectomy are additive in grasshoppers, with the combination of the two treatments resulting in a longer lifespan than either treatment alone. This suggests separate mechanisms for these two routes to life extension (Drewry et al. 2011).

In the present study, we measured the degree to which ovariectomy reduced feeding rates of grasshoppers, and then offered this same feeding rate to sham-operated grasshoppers. In this way, we created a dietary restricted group with feeding matched to the reduced reproduction group. This allows direct comparison of the effects of reproduction on physiological parameters associated with longevity, without the potential confounding effects of differences in feeding. We chose several physiological measures, focusing on energy balance. First, we measured the size of organs known as protein and fat depots in grasshoppers, as metrics of the storage of the limiting nutrient (protein) and stored energy (fat). The hemolymph volume serves as a proxy for protein storage, as insects store high concentrations of amino acids in the hemolymph as hexameric proteins, and these are generally considered to be the major depot of amino acids in insects (Nijhout 1994). Next, we measured total anti-oxidant activity in muscle and hemolymph, because avoiding oxidative damage has long been thought to be an important mechanism of life extension. Third, numbers of mitochondria are increased in many animals upon life-extending dietary restriction (Guarente 2008), and this can be taken to indicate a shift toward more efficient use of energy. Last, we measured the transcript abundances of two proteins known to be associated with reproduction in grasshoppers, namely vitellogenin and the hexamerin-90 storage protein (e.g., Hatle et al. 2001), to determine the degree of reduction of reproduction and storage.

We found that ovariectomized grasshoppers had greatly increased organismal storage in comparison to matched-fed grasshoppers on dietary restriction. In contrast, these two groups had similar anti-oxidant activities, similar mitochondrial counts, and similar transcript levels of storage protein.

Methods

Surgeries and Diets

Juvenile *Romalea microptera* were obtained from Miami, FL, USA as in Hatle et al. (2008) and were kept *en masse* and fed Romaine lettuce *ad libitum*. Adult females were separated and reared individually on a 14L:10D photoperiod and a corresponding 35°C:27°C thermocycle. On the day of adult molt, individuals were serially assigned to one of three treatment groups: sham-operated & full diet (Sham-FD, n = 30), ovariectomized & full diet (OVX-FD, n = 24), or sham-operated & dietary restriction (Sham-DR, n = 25). Ovariectomies and sham (control) operations were performed within the first 3 days of adulthood as described previously (Hatle et al. 2003). All individuals were fed daily, and survivorship was recorded daily. Full diet animals were fed Romaine lettuce *ad libitum*. About every 7 d, the amount of Romaine lettuce eaten by the OVX-FD individuals was quantified (as in Drewry et al. 2011). The mean amount eaten daily by the OVX-FD individuals in the previous week interval was then offered to the Sham-DR group in the following week.

Starting at approximately age 30 d, all individuals were placed on sand two or three times a week to allow for oviposition of eggs, as retaining unlaid eggs can affect the physiology of

the female (cf. Hatle et al. 2008 and Drewry et al. 2011). Any females that attempted to probe into the sand and lay eggs were left undisturbed overnight.

Sample Collection

Samples were collected at two distinct age blocks. About one-third of the surviving animals were sampled when survivorship in the Sham-FD group was ~85% (mean $\pm$ SE = 83.4 $\pm$ 0.3; range 80–86 d). The remaining surviving animals were sampled when survivorship in the Sham-FD group was ~50% (mean $\pm$ SE = 135.1 $\pm$ 0.7; range 124–141 d). Hereafter, these cohorts will be called “85 d” and “135 d” respectively.

During sample collection, one hindleg was removed and the animal was bled nearly completely; while this is not a fully quantitative method of measuring hemolymph volume, it provides a sound relative measure of hemolymph volume (as in Judd et al. 2011). Five microliters of this hemolymph sample was transferred to 250 μ l of phosphate buffered saline (PBS). Then the abdomen was opened and the fat body was removed, the mandibular skeletal muscle was removed from the head, and the skeletal muscle was scrapped out of one femur. All these samples were immediately weighed in tared tubes, frozen in liquid nitrogen, and then kept frozen until analysis.

Sample Analysis

Anti-oxidant activities – Both mandibular muscle and hemolymph samples were tested for total anti-oxidant power by the 2, 2' – azino – bis(3 – ethylbenzothiazoline – 6 – sulphonic acid) (ABTS) decolorization assay, as trolox equivalents (see Drewry et al. 2011; after Re et al. 1999). The mandibular muscle was bead homogenized in PBS, tested for total protein by the Bradford (1976) assay, and then the volume containing 50 μ g of protein was assayed for anti-oxidant activity. Similarly, the raw hemolymph sample was thawed, suspended in PBS, assayed for total protein, and then the volume containing 50 μ g was assayed for anti-oxidant activity.

Catalase activity (mU/mg protein) was measured with the hemolymph sample stored in PBS using the Amplex® Red Catalase Assay Kit, a chemical probe system in Tris – HCl buffer, from Invitrogen (Eugene, OR, USA).

Mitochondrial Counts – Mitochondrial counts were estimated as the molar ratio of cytochrome B, a mitochondrial gene, relative to beta-actin, a nuclear gene (e.g., Bogacka et al. 2005). Total genomic DNA was extracted from femur muscle and fat body using the DNeasy Blood and Tissue Kit from Qiagen (Valencia, CA, USA); this extraction isolates only DNA, not RNA. We found that femur muscle samples did not yield usable DNA when digested in proteinase K overnight, but 2 hr digestions produced good product. Genomic DNA was stored at –20°C or 4°C, but only thawed once.

A 420 bp region of cytochrome B from lubber grasshoppers has been sequenced previously to examine population variation (Mutun and Borst 2004). From this sequence we developed new primers using the Primer3 software: Left, 5' TCC TTT TGA GGT GCA ACA GT; Right, 5' GAT CCT GTT TGG TGG AGG AA. This cytochrome B primer set was used for qPCR at 10 μ M (final concentration of 1 μ M). For actin, we used the primer set from Fei et al. (2005) at 1.5 μ M. Thermocycling parameters for RT-PCR were: 95°C for 3 min, followed by 35 cycles of 95°C, 49°C, and 72°C for 30 sec each. We used iQ SYBR Green Supermix with a MiniOpticon RT-PCR system (Bio-Rad, Hercules, CA, USA). Products were checked for purity via melting curves and agarose gel electrophoresis. Standard curves for absolute quantification were created by making standards from purified PCR products. Concentrations of the PCR products were determined by spectrophotometry, and standards were stored at –20°C. Ten-fold serial dilutions of these standards were run with each batch

of unknown samples. The log₁₀ of the cross threshold (C_t) values for each set of standards were plotted against the amount of DNA in each standard to produce linear standard curves. The molar concentration in each standard was estimated assuming each base pair was 675 Da. The relative number of mitochondria in each sample was expressed as the molar ratio of cytochrome B to genomic actin.

Transcript levels – Messenger RNA was extracted from the same femur muscle and fat body samples used for mitochondrial counts, and the RNA extractions were performed simultaneously with the DNA extractions so that samples were only thawed once. Extractions were done with the Ambion RiboPure kit (Invitrogen, Grand Island, NY, USA). RNA was stored at -20°C or 4°C and then converted to cDNA libraries with the Bio-Rad (Hercules, CA, USA) iScript cDNA synthesis kit. cDNA libraries were stored at -20°C or 4°C , but were only thawed once.

Vitellogenin and hexamerin-90 transcript levels, relative to beta-actin, were quantified by qRT-PCR using previously published primers (Fei et al. 2005, Hathaway et al. 2009). Thermocycling parameters were: 95°C for 3 min, followed by 35 cycles of 95°C , 49°C , and 72°C for 30 sec each. Standard curves were run as for the determination of mitochondrial counts. The molar concentration of each unknown (for both vitellogenin and hexamerin-90) was expressed relative to the molar concentration of actin for that sample.

Statistical analyses

All analyses except survivorship were run in SAS as in previous work (SAS Institute Inc., 1999; Drewry et al. 2011). Data were checked visually for normality.

Feeding rates were tested using a one-way MANOVA with time as a dependent variable; feeding rates were not statistically compared after 80 d due to declining sample sizes. Median ages at death were compared using the Kaplan-Meier time-failure analysis, implemented in Excel (e.g., Lee and Wang 2003, as in Hatle et al. 2006b).

The effects of treatment (i.e., Sham-FD, OVX-FD, or Sham-DR) and age on organismal storage (as hemolymph volumes and fat body masses) were tested using a two-way MANOVA with Ryan-Einot-Gabriel-Welsch Q (REGWQ) post-tests.

The effects of treatment and age on anti-oxidant activities were tested with three separate two-way ANOVAs (one for mandibular muscle, one for hemolymph, and one for catalase) with Tukey-Kramer post-tests. These were run separately to maximize the samples sizes, because we were not able to get data for all three assays for each individual. We also tested the combined data as a single two-way MANOVA, which resulted in using a much lower sample size (total $n = 26$). The qualitative results for the single MANOVA were the same as those for the three separate ANOVAs.

For mitochondrial counts, we analyzed the effects of treatment (Sham-FD, OVX-FD, and Sham-DR), age (85 d or 135 d), and tissue (femur muscle or fat body) using a three-way ANOVA with Tukey-Kramer post-tests. To determine the role of treatment in the significant effects, we dropped tissue (which was clearly dominant) from the analysis and then compared F-values to estimate the percentage contribution of treatment to the significant effect.

The effects of treatment and age on transcript levels for vitellogenin and hexamerin-90 in the fat body were compared using 2-way MANOVA with Tukey-Kramer post-tests.

Results

Matched diets for OVX-FD and Sham-DR

In early adulthood, there was no difference in feeding rates between fully reproductive control animals and ovariectomized animals. Feeding rates after 40 d (the age at which the control group laid their first clutch of eggs) dropped substantially in ovariectomized females (Hatle et al. 2008; Drewry et al. 2011). OVX-FD and Sham-FD had feeding rates (Figure 1) that were not significantly different on days 4 ($F_{1,46} = 0.97$, $P = 0.330$), 10 ($F_{1,46} = 2.79$, $P = 0.102$), 17 ($F_{1,46} = 3.32$, $P = 0.075$), and 31 ($F_{1,46} = 0.48$, $P = 0.492$). Feeding rates were significantly different on days 38 ($F_{1,46} = 20.73$, $P < 0.0001$), 52 ($F_{1,46} = 12.38$, $P = 0.001$), 59 ($F_{1,46} = 19.03$, $P < 0.0001$), 66 ($F_{1,46} = 6.64$, $P = 0.013$), 73 ($F_{1,46} = 4.34$, $P = 0.043$), and 80 ($F_{1,46} = 16.98$, $P = 0.0002$).

The food offered to the Sham-DR group was matched to that consumed by the OVX-FD group. To ensure this feeding level was a restricted diet, we checked the frequency of completion of the daily meal in the Sham-DR group. From median ages 44 through 60 d, Sham-DR individuals completed their meals 97% of the time, suggesting the food offered was limiting. Further confirming the limitation of the Sham-DR feeding, the number of eggs per clutch was not reduced in clutch 1 ($P = 0.302$), when there was no restriction compared to Sham-FD controls. In contrast, the number of eggs laid by the Sham-DR group was clearly reduced in clutch 2 ($P = 0.001$) and clutch 3 ($P < 0.0001$), when feeding was restricted compared to Sham-FD controls (data not shown; consistent with Drewry et al. 2011).

Increased survivorship in both OVX and DR

Survivorship was greatly increased by either ovariectomy or dietary restriction. Kaplan-Meier survivorship tests showed that OVX-FD ($\chi^2 = 48.6$, $df = 1$, $P < 0.001$) and Sham-DR ($\chi^2 = 98.9$, $df = 1$, $P < 0.001$) females had significantly higher survivorship than Sham-FD females (Figure 2). In contrast, OVX-FD and Sham-DR females barely differed in survivorship ($\chi^2 = 4.0$, $df = 1$, $P < 0.025$).

Nutrient storage

Ovariectomized & full diet females had much greater fat body masses and hemolymph volumes than Sham-FD and Sham-DR females. Fat body mass and hemolymph volume are important metrics for storage of lipid and protein, respectively, in grasshoppers. These were the only measures in the study for which the direct comparison of the OVX-FD and Sham-DR groups produced a statistically detectable difference. Hemolymph volume was significantly affected by treatment ($F_{2,77} = 23.8$; $P < 0.0001$) and age ($F_{1,77} = 4.32$; $P = 0.041$), but not by the interaction of treatment and age ($F_{2,77} = 2.01$; $P = 0.14$; Figure 3). The age effect was marginal, as the REGWQ post-test showed ages 85 d and 135 d as not statistically different. For analysis of fat body wet mass, there was a highly significant effect of treatment ($F_{2,77} = 46.7$; $P < 0.0001$), but no effect of age ($F_{1,77} = 1.4$; $P = 0.239$) or the interaction ($F_{2,77} = 0.34$; $P = 0.71$; Figure 3). The effect sizes of the response variables (as R^2 in the model) were 0.60 for fat body mass and 0.48 for hemolymph volume.

Anti-oxidant activities

Treatment did not affect anti-oxidant activities in any tissue, while age had an effect in one case. Specifically, total anti-oxidant activity in mandibular muscle was not affected by treatment ($F_{2,47} = 0.48$, $P = 0.622$), age ($F_{1,47} = 3.33$, $P = 0.075$), or their interaction ($F_{2,47} = 0.30$, $P = 0.744$, Figure 4). Total anti-oxidant activity in the hemolymph also was not affected by treatment ($F_{2,59} = 2.23$, $P = 0.118$) but was higher at 85 d than at 135 d ($F_{1,59} = 11.43$, $P = 0.0013$). There was no detectable interaction between age and treatment on

hemolymph total anti-oxidant activity ($F_{2,59} = 2.41$, $P = 0.100$). The activity of one important anti-oxidant enzyme in the hemolymph, catalase, was not affected by treatment ($F_{2,57} = 1.23$, $P = 0.302$), age ($F_{1,57} = 3.51$, $P = 0.067$), or their interaction ($F_{2,57} = 1.39$, $P = 0.258$). The overall effect size (i.e., R^2) for anti-oxidant activities was 0.26.

Mitochondrial numbers

Numbers of mitochondria were estimated by measuring the number of copies of a mitochondrial gene relative to the number of copies of a nuclear gene. Mitochondrial counts were strongly affected by tissue, with many more mitochondria in femur muscle than in fat body (Pillai's Trace, $F_{1,64} = 198.1$, $P < 0.0001$), affected by age ($F_{1,64} = 13.0$, $P = 0.0006$), with more mitochondria at 85 d than at 135 d, and weakly but significantly affected by treatment ($F_{2,64} = 3.6$, $P = 0.035$, Figure 5). The overall effect size (i.e., the R^2 of the model) 0.79.

Effects of treatment (i.e., Sham-FD, OVX-FD, or Sham-DR) contributed ~20% of the total variance in mitochondrial numbers that were explained by the model. Hence, our manipulative treatments were not as influential as age, but our treatments still had clearly detectable effects on mitochondrial number. The pairwise comparisons showed that the Sham-FD group had significantly more mitochondria than the OVX-FD group ($P = 0.027$) but not the Sham-DR group ($P = 0.563$). The pairwise comparison showed no detectable difference in mitochondrial numbers between the OVX-FD group and the Sham-DR group ($P = 0.249$).

There was no interaction between treatment and age ($F_{1,64} = 1.4$, $P = 0.246$), but there were clear interactions between tissue and age ($F_{1,64} = 8.4$, $P = 0.005$), and treatment and tissue ($F_{2,64} = 5.1$, $P = 0.009$). This treatment by tissue interaction was significant because the difference in mitochondrial counts between the fat body and femur muscle was smaller for the OVX-FD group than for the two sham-operated groups. There was no detectable three-way interaction among treatment, age, and tissue ($F_{2,64} = 1.25$, $P = 0.295$).

Fat body transcript levels

Vitellogenin transcript levels were affected by treatment ($F_{2,35} = 5.10$, $P = 0.012$). Pairwise comparisons showed that fat bodies of the Sham-FD group had significantly higher vitellogenin transcript levels than the OVX-FD group ($P = 0.013$), but only marginally higher levels of vitellogenin transcripts than fat bodies of the Sham-DR group ($P = 0.069$). Vitellogenin transcript levels differed with age ($F_{1,35} = 5.63$, $P = 0.024$), but this age effect varied across treatment groups producing a significant age by treatment interaction ($F_{2,35} = 4.65$, $P = 0.018$). Overall, vitellogenin transcript levels were highest in Sham-FD females at 85 d. The expression of vitellogenin transcript was consistent with reduced reproduction in the OVX-FD and Sham-DR groups. In contrast, hexamerin-90 transcript levels were not affected by treatment ($F_{2,35} = 1.62$, $P = 0.215$), age ($F_{1,35} = 0.48$, $P = 0.495$), or their interaction ($F_{2,35} = 2.85$, $P = 0.073$). The effect size of the model (i.e., R^2) was 0.46.

Discussion

Ovariectomized & full diet grasshoppers and dietary restricted grasshoppers with matched feeding rates had similar increases in survivorship in comparison to sham-operated & full diet controls. In comparing the OVX-FD and Sham-DR groups, the most conspicuous physiological difference was that the OVX-FD group had much greater fat body masses and hemolymph volumes. In contrast, anti-oxidant activities, mitochondrial counts, and vitellogenin and hexamerin-90 transcript levels did not differ between the OVX-FD and Sham-DR groups. Hence, when feeding rates were controlled, ovariectomy and dietary

restriction showed large differences in nutrient storage but no observed differences in cellular hallmarks of aging.

Ovariectomy reduces feeding to a degree that is sufficient to enhance survivorship in intact controls

In the present study, we confirmed that ovariectomy in grasshoppers reduces feeding rate after ~40 d to about 60% of that eaten by controls (Drewry et al. 2011; Judd et al. 2011), and starting dietary restriction at 50 d after adult molt extends lifespan in grasshoppers (Hatle et al. 2006b). Hence, our results in this experiment for the longevity-extending effects of ovariectomy or dietary restriction were consistent with previous studies. The present study substantially extends our previous work by using matched feeding levels to show that the degree and timing of reduced feeding associated with ovariectomy is sufficient to increase survivorship in Sham-DR animals. Both the OVX-FD and the Sham-DR groups had increased survivorship, with nearly 70% survivorship at 120 d in comparison to <50% survivorship for the Sham-FD control group.

Ovariectomy and dietary restriction in grasshoppers are two distinct routes to life extension. Drewry et al. (2011) showed that the life-extension from ovariectomy and dietary restriction is additive; that is, ovariectomized females fed limited quantities (eating all of their daily meals ~90% of the time) lived longer than ovariectomized females fed *ad libitum*. Further, in our previous studies ovariectomy greatly increased hemolymph vitellogenin levels, whereas dietary restriction did not. Drewry et al. (2011) concluded that the physiology underlying ovariectomy must be different from that of dietary restriction, but had no information on storage or cellular parameters. The matched feeding rates used in the present experiment (i.e., the comparison of the OVX-FD and the Sham-DR) allow a physiological analysis of some potential mechanisms, without the confounding variable of feeding level.

Ovariectomy greatly increases organismal storage

The OVX-FD group had at least 2-fold higher fat body masses and hemolymph volumes than either the Sham-FD group or the Sham-DR group. Surprisingly, these increases in organismal storage were the only statistically detectable differences between the OVX-FD and Sham-FD groups in this study. In insects, the fat body is the main organ for storage of lipids (similar to the adipose in vertebrates), as well as serving as the main metabolic organ (similar to the liver in vertebrates). The hemolymph is generally considered the main depot of stored protein, in the form of hexamerins (e.g., Nijhout 1994). In previous work, we have shown that ovariectomy mildly increased hemolymph levels of all three grasshopper hexamerins combined (Hatle et al. 2008). Therefore, in the present study ovariectomized grasshoppers showed large magnitude increases in organismal storage of both protein and lipid. In contrast, the ~50% difference in feeding rates between the Sham-FD and Sham-DR groups did not result in differences in fat body masses and hemolymph volumes. The fact that such a drastic dietary treatment failed to generate statistically significant differences in organismal storage underscores the dramatic impact of ovariectomy on increasing storage.

Mitochondrial numbers were also consistent with a shift toward greater organismal storage upon ovariectomy. Both production of and maintenance of mitochondria are energetically expensive (e.g., Guarente 2008; Lopez-Lluch et al. 2005), so fewer mitochondria would be in line with a shift in investment toward storage. Indeed, OVX-FD females had fewer mitochondria than Sham-FD controls, largely due to differences in skeletal muscle in the femur. Near as we can tell, fewer mitochondria in the muscle cells of OVX-FD females could allow an overall savings in energy, permitting greater storage.

Increased fat storage upon a life-extending extension of reproduction has been observed in both *C. elegans* (O'Rourke et al. 2009) and *Drosophila* (Flatt et al. 2008), and it is consistent with increased adiposity upon gonadectomy in mammals (reviewed in Hansen et al. 2013). In this context, the increase in fat body mass in this study is not surprising. However, no other study in invertebrates has reported reduced feeding rates and simultaneous increased fat storage in concert with longevity, in comparison to fully reproductive controls.

Ovariectomy & dietary restriction differ little in anti-oxidant activities and mitochondrial numbers

Life-extending dietary restriction is associated with both higher anti-oxidant activities and increased numbers of mitochondria in several animal models (e.g., Muller et al. 2007 and Guarente 2008 respectively). For anti-oxidant activity in particular, some results in rodents are inconsistent with the oxidative stress hypothesis (Jang et al. 2009; Zhang et al. 2009), but other work suggests oxidative damage can be important in aging (Williams et al. 2009; Salmon et al. 2010; Kirkwood and Kowald 2012). Therefore, we predicted that anti-oxidant activities and mitochondrial numbers would be higher upon dietary restriction than upon ovariectomy. However, neither parameter differed in direct comparison between the OVX-FD group and the Sham-DR group.

Because we detected clear differences in total anti-oxidant activity with age, wherein young animals had greater activities than old animals, our assay was sufficiently sensitive to reveal physiological differences. That said, the degree to which our multivariate statistical model explains the variability in anti-oxidant activities was notably less than the other statistically significant physiological data in this study (see effect sizes in Results). This indicates that although age significantly affected total anti-oxidant activity in the hemolymph, it had only a weak effect. The overall picture is that either life-extending treatment had little effect on anti-oxidant activities.

There was no detectable difference in mitochondrial numbers between the OVX-FD group and the Sham-DR group. It is somewhat surprising that dietary restriction did not increase mitochondrial counts, because this has been observed in several other models of aging (Guarente 2008). A complete assessment of mitochondrial function should include estimates of both mitochondrial number and respiratory activity (Lopez-Lluch et al. 2006). It may be that dietary restriction altered mitochondrial function via a change in activity instead of changing mitochondrial abundance. That said, the present data suggests that OVX-FD and Sham-DR grasshoppers are unexpectedly similar in mitochondrial numbers.

Increased fat body mass means increased organismal levels of transcripts

Because fat body mass was increased upon ovariectomy, organismal levels of vitellogenin and hexamerin-90 transcript for the OVX-FD are higher than the mass specific values in Fig. 6 (relative to the Sham-FD and Sham-DR groups). This is because fat body is the main metabolic organ in insects, not merely a lipid depot (Nijhout 1994). For plasticity in reproduction, we showed that the mass specific production rate of vitellogenin was not as important as the increase in fat body mass to the changes in total (i.e., organismal) vitellogenin synthesis (Hatle et al. 2006a). In the present paper we see a similar effect, namely that the magnitude of the differences in fat body mass results in a greater number of total transcripts, particularly at 135 d. It may be that this long-lived, fully-fed group is better maintaining production of these proteins. Perhaps more important, the protein production role of the fat body highlights the importance of changes in size of this metabolic organ. Indeed, signals from growth factors (e.g., insulin-like peptides) could increase the size of the organ, resulting in greater production of multiple products.

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Highlights

>Ovariectomy reduced feeding in grasshoppers, and offering this feeding rate to intact females results in increased survivorship. >Ovariectomy doubles nutrient storage. >Ovariectomy does not alter anti-oxidant activity or mitochondrial counts, in comparison to matched-fed controls. >Reduced reproduction can simultaneously decrease feeding, increase lifespan, and increase fat storage.

Figure 1.

LSMeans $\pm$ SEs of lettuce consumed per day (total $n = 48$). Ovariectomized & full diet (OVX-FD) grasshoppers consumed less lettuce than sham-operated & full diet (Sham-FD) grasshoppers after ~40 d, the approximate age at first oviposition in controls. Sham-operated & dietary restricted (Sham-DR) grasshoppers were offered daily meals equal to the amount consumed by OVX-FD grasshoppers, and they ate all of it >90% of the time.

Figure 2.

Both ovariectomized & full diet (OVX-FD) and sham-operated & dietary restricted (Sham-DR) grasshoppers had better survivorship than sham-operated & full diet (Sham-FD) controls. Total n = 120.

Figure 3.

LSMeans $\pm$ SEs of storage parameters in grasshoppers. Ovariectomized & full diet (OVX-FD) grasshoppers had greatly increased organismal storage relative to both sham-operated & full diet (Sham-FD) and sham-operated & dietary restricted (Sham-DR) grasshoppers.

Hemolymph in grasshoppers is a major storage site for protein (in the form of hexameric storage proteins) while fat body is both a major lipid depot and a metabolic organ. Total $n = 79$.

Figure 4.

Anti-oxidant activities (LSMeans $\pm$ SEs) from various tissues of grasshoppers were not different among sham-operated & full diet (Sham-FD), ovariectomized & full diet (OVX-FD), or sham-operated & dietary restricted (Sham-DR) grasshoppers. Total anti-oxidant activity from the hemolymph was significantly greater at 85 d than at 135 d. Total anti-oxidant activity is presented as equivalents of trolox (mM) / protein (ug). Catalase activity is presented as mU / protein (mg). Total n = 49.

Figure 5.

Numbers of mitochondria (LSMeans $\pm$ SEs) in grasshoppers were significantly higher in femur muscle than in fat body and higher at 85 d than at 135 d. Numbers of mitochondrial were slightly higher in sham-operated & full diet (Sham-FD) females than ovariectomized females & full diet (OVX-FD) females, but not than sham-operated & dietary restricted (Sham-DR) females. Numbers of mitochondria were estimated by measuring the copies of a mitochondrial gene (cytochrome B) relative to the copies of a nuclear gene (beta-actin). Total n = 66.

Figure 6.

Levels in the fat body of transcripts (LSMeans $\pm$ SEs) related to reproduction in lubber grasshoppers. At 85 d, sham-operated & full diet (Sham-FD) females had significantly higher vitellogenin transcript levels than did ovariectomized & full diet (OVX-FD) females and marginally higher levels than did sham-operated & dietary restricted (Sham-DR) females. Levels of vitellogenin transcript at 135 d were not affected by treatments. Levels of transcript for hexamerin-90 (a storage protein) were affected by neither treatment nor age. Total n = 36.