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

John D. Hatle [a,*], James W. Kellenberger [a], Ephraim Viray [a], Alicia M. Smith [a], Daniel A. Hahn [b]

[a] University of North Florida, 1 UNF Drive, Department of Biology, Jacksonville, FL 32224, USA
[b] The University of Florida, Department of Entomology and Nematology, PO Box 110620, Building 970 Natural Area Drive, Gainesville, FL 32611, USA

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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.

1. 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 *Caenorhabditis elegans* results in the 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

* Corresponding author. Tel.: +1 904 620 2778; fax: +1 904 620 3885.
E-mail address: jhatle@unf.edu (J.D. Hatle).

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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 and Hatle, 2008; Judd et al., 2011). This species of grasshopper has potent chemical defenses (e.g., Hatle et al., 2002a, 2002b), 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., 2002a) 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 an ~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 the 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 the 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.

2. Methods

2.1. Surgeries and diets

Juvenile *R. 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 d 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 the oviposition of eggs, as retaining unlaid eggs can affect the physiology of the female (cf. Drewry et al., 2011; Hatle et al., 2008). Any females that attempted to probe into the sand and lay eggs were left undisturbed overnight.

2.2. 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 ± SE = 83.4 ± 0.3; range 80–86 d). The remaining surviving animals were sampled when survivorship in the Sham-FD group was ~50% (mean ± SE = 135.1 ± 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 μl 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.

2.3. Sample analysis

2.3.1. 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 μg 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 μg 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).

2.3.2. Mitochondrial counts

Mitochondrial counts were estimated as the molar ratio of cytochrome B, a mitochondrial gene, relative to beta-actin, a nuclear gene