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Life-extending dietary restriction and ovariectomy result in similar feeding rates but different physiologic responses in grasshoppers

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ABSTRACT

Dietary restriction (DR) and reduced reproduction each extend life span in many species. Females undergoing DR typically experience a reduction in their fecundity, which raises the question of whether the two treatments are actually extending life span in overlapping ways. Life span in lubber grasshoppers has been shown to be increased by DR, and separately by ovariectomy (OVX). Here, we test the combination of these on life span. If life extension by the two treatments are additive, it would suggest that they likely act through separate pathways. The experimental groups were: fully reproductive and fully fed (ShamFD); ovariectomized and fully fed (OVX FD); fully reproductive and restricted diet (ShamDR); and ovariectomized and restricted diet (OVX DR). The median life spans of these groups were: ShamFD = 245 d, OVX FD = 285 d, ShamDR = 286 d, and OVX DR = 322 d. Feeding rate for the OVX FD group was 64% of ad libitum, similar to the 70% of ad libitum that was used for ShamDR. We also measured hemolymph parameters of physiology in these same individuals. Hemolymph levels of vitellogenin (the egg yolk-precursor protein) were increased 5-fold by OVX, but were not affected by DR. In addition, hemolymph total anti-oxidant activity (per μg protein) was significantly reduced by OVX, but was not affected by DR. We show that OVX and DR produce different physiological responses in grasshoppers, despite life extensions and feeding levels that were not significantly different. These data suggest that OVX and DR might extend life span via distinct pathways.

1. Introduction

Dietary restriction (DR) and reduced reproduction each extend life span in many animals, but the relationship between the two treatments is unclear. Many animals undergoing DR also experience a reduction in their fecundity (as reviewed in Partridge et al., 2005; detailed in Carey et al., 2008). This has led to the question of whether the two treatments are actually extending life span in similar ways (e.g., Crawford et al., 2007). This can be addressed in part via a fully factorial experiment that tests whether animals subjected to both DR and directly reduced reproduction live longer than animals on either treatment alone. If the life extensions due to DR and reduced reproduction are additive, it would suggest that they act through separate mechanisms. This conclusion could be supported further by different physiological responses to DR and reduced reproduction, addressing several of the concerns laid out by Gems et al. (2002).

The fact that DR typically reduces fecundity implies that life extension via either treatment may be due simply to reduced reproduction. For instance, in *C. elegans*, removal of the germline stem cells nearly doubled life span, and applying DR to worms with the germline ablated did not further extend life span. The authors conclude the life extension pathways of DR and reduced reproduction are at least partly overlapping in worms (Crawford et al., 2007).

On the other hand, some studies have concluded that life span extension by DR does not act through reduced reproduction (e.g., Mair et al., 2004; reviews in Barnes and Partridge, 2003; Koubova and Guarente, 2003; Kenyon, 2005; Partridge et al., 2005). In *Drosophila melanogaster*, DR was combined with experimentally suppressed reproduction. The suppression of reproduction was accomplished in three ways: via X-irradiation, which sterilized the germline; segregating females from males to remove the stress of mating; or by the ovo^{DI} mutation, which blocks ovarian development and vitellogenesis. Dietary restriction extended the life span of all groups of female flies regardless of experimental manipulation of reproduction. This suggests that life span extension via DR in female *D. melanogaster* is not directly linked to reproductive activity (Mair et al., 2004).

While many studies have manipulated diet and measured fecundity, few studies have manipulated reproduction and measured individual feeding rates (similar to Shimokawa et al., 2003 for dietary restriction and growth hormone knockdown in mice). Measuring ingestion in fruit flies is contentious (but see Lee et al., 2008), yet it is important because of the possibility of compensatory feeding on a low quality diet (Carvalho et al., 2005; Mair et al., 2005; Min and Tatar, 2006; Min et al., 2007). Measurements of ingestion rate in *C. elegans*

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have never been published, to our knowledge. In contrast to fruit flies or worms, the relatively large size of our experimental model, the lubber grasshopper, makes measuring individual feeding levels possible. This allows observations on the associations among food consumption, reproduction, and life span, while simultaneously tracking physiological responses in the hemolymph. Here, we address whether dietary restriction and reduced reproduction extend life span via overlapping pathways in grasshoppers.

In previous work, DR greatly extended life span in lubber grasshoppers, without changing life-time fecundity (Hatle et al., 2006). Further, DR (70% of ad libitum feeding) had no significant effect on storage protein levels in the hemolymph, a major depot for reproduction in this phytophagous insect (Hatle et al., 2001). However, the study design in Hatle et al. (2006) did not allow oviposition of eggs into suitable substrate, and this may have affected the physiology of the females.

In a separate study, ovariectomy extended life span in grasshoppers by ~22% without any difference in the amount of food ingested (Hatle et al., 2008). As with the grasshopper DR study, this design did not allow females access to oviposition substrate, which may have affected the physiology of controls. In contrast to other aging models, ovariectomy in grasshoppers only prevents the formation of eggs. Specifically, the egg yolk-precursor protein vitellogenin is produced, but because the ovary is missing, the vitellogenin accumulates to high levels in the hemolymph (Hatle et al., 2003). Hence, ovariectomy in grasshoppers permits the allocation of a limiting nutrient (viz., protein) to an early stage of reproduction. In concert with the increased life span upon ovariectomy, levels of hemolymph storage proteins were significantly higher within old ovariectomized females (Hatle et al., 2008). This is different from the lack of response of storage proteins to DR, and these contrasting results suggest that DR and ovariectomy may have different effects on the physiology of females.

In the present study, we address whether life extensions via DR and reduced reproduction act through similar mechanisms. In particular, DR and ovariectomy are combined to test whether the increased longevity due to the two treatments is additive. Feeding rates are simultaneously measured to determine whether they play a role in life extension upon reduced reproduction. Further, total anti-oxidant activity in the hemolymph and vitellogenin are measured to address whether these physiological responses to DR and ovariectomy are the same.

2. Methods

2.1. Surgery, diet, and data collection

Juvenile *Romalea microptera* were obtained from Miami, FL, USA as in Hatle et al. (2008) and were kept en masse and fed ad libitum Romaine lettuce. Adult females were separated and serially assigned to either a fully fed, ad libitum diet (FD) or a restricted diet (DR). In most animals that have been tested, life span increases until the point of starvation (~35% ad libitum), and an effective, life-extending quantity of DR is ~70% of ad libitum (Weindruch and Walford, 1988; Hatle et al., 2006). Hence, our DR treatment was 70% that of the amount eaten daily by the FD group. Approximately every 10 d, the Romaine lettuce left uneaten from the previous day by the FD individuals was collected and dried overnight at 55 °C. A conversion for dry mass from wet mass was calculated from known amounts of fresh lettuce and used to determine average amount eaten daily (Hatle et al., 2006). Within these dietary regimens, females were serially assigned to ovariectomy (OVX), or a control operation (Sham), leading to a fully factorial 2×2 design of Sham FD, OVX FD, ShamDR, and OVX DR (n = 40 per group). All four groups were fed their dietary regimen daily and kept in individual ventilated containers of approximately 500 cm^3 on a 14 L: 10D photoperiod at 32 °C during the day and 24 °C at night. The study was terminated at 365 d.

Ovariectomies and sham (control) operations were performed on the first day after adult molt as previously (Hatle et al., 2003). Starting at approximately age 30 d, all sham and ovariectomized individuals were placed on sand two times a week to allow for oviposition of eggs (Hatle et al., 2001). Any females that attempted to probe into the sand and lay eggs were left undisturbed overnight at room temperature. Clutches of eggs were collected and total number of eggs, clutch number, and age at oviposition were recorded. Previous work has shown that reduced diet has little or no effect on egg size (Moehrlin and Juliano, 1998). At time of death, any melanization of the ovaries was recorded. Melanization is an immune response to infection; in the ovary, it causes a black, hardened growth which encloses the ovarioles and unlaid eggs and prevents further oviposition (Beckage, 2008).

2.2. Hemolymph total anti-oxidant activities and vitellogenin levels

For about one-quarter of the animals in the study, hemolymph samples were collected monthly from about four months until the end of the experiment, using established methods (e.g., Hatle et al., 2006). For anti-oxidant activity, a 1 µl sample was flash frozen in liquid nitrogen and stored at −20 °C for later assay (Judd et al., 2010). Activity was measured as the reduction of trolox in an aqueous buffer (after Re et al., 1999, see also Williams et al., 2008), so this assay detects only water-soluble anti-oxidants. For vitellogenin measurement, a 5 µl hemolymph sample was placed in 250 µl of phosphate buffered saline and stored at −20 °C and later assay by ELISA as described (Borst et al., 2000; Hatle et al., 2001; 2004; 2008).

2.3. Statistics

The amount of food eaten at each sampling date for FD groups (Sham and OVX) was compared by a MANOVA with time as a dependent variable (a type of repeated measures analysis). Similarly, for all groups, body masses of each individual over time were compared with a MANOVA. Vitellogenin levels were also tested using two-way MANOVA with time as a dependent variable, while total anti-oxidant activities were tested with a two-way ANOVA. The assumptions underlying all MANOVA and ANOVA analyses were not tested, but the relatively large sample sizes for most analyses in this study means they are nonetheless likely to be robust. Further, we used the test statistic for MANOVA (i.e., Pillai's Trace) that is the most robust for deviations from the assumptions (Scheiner, 1993).

For Sham reproductive data, MANOVA was used to assess eggs per clutch and time between clutches. Student's *t*-tests were performed for total fecundity (cumulative number of eggs), average eggs per clutch, average time between clutches, and average number of clutches per group. Because multiple *t*-tests were used, a Bonferroni correction of alpha was used, which was 0.05/4 = 0.0125. The rates of melanization were compared using an r×c chi-squared test for association with one degree of freedom (Holmes et al., 2006). All MANOVAs were performed in SAS (SAS Institute, 2009) and all *t*-tests were done in Microsoft Excel®. An estimate of Lifetime Reproductive Effort (as in Charnov et al., 2007) was calculated using the following formula: (clutches)×(clutch size)×(average adult life span)×(offspring mass at independence)/(adult mass at first reproduction).

Survivorship curves were analyzed using Cox proportional hazards models implemented with Proc PHREG in SAS (SAS Institute, 2009). The proportional hazards model is the preferred method in survival analysis, and it can tolerate censored individuals (Cox, 1972; Allison, 2010). Important for testing our questions, proportional hazards models allow testing of a possible interaction of diet and surgery. A significant interaction would indicate that the relationship differs significantly from an additive response (e.g., it is either synergistic or antagonistic). In contrast, a non-significant result would suggest a mathematically additive response to the two treatments. Maximum likelihood estimates approximate the contribution of the treatment to