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Knockdown of vitellogenin by RNAi increases survivorship but exhibits similar physiological responses to ovariectomy in grasshoppers

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Knockdown of vitellogenin by RNAi increases survivorship but exhibits similar physiological responses to ovariectomy in grasshoppers

by

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Table of Contents

List of Figures

Abstract

Reduced reproduction has been shown to increase lifespan in many animals, yet the mechanisms behind this trade-off are mostly unknown. A previous study has shown that in the lubber grasshopper, *Romalea microptera*, ovariectomized (OVX) individuals have a 30% increase in lifespan relative to controls (Sham). In a separate study, an increase in fat body mass and a halting of ovarian growth were seen upon reduction of vitellogenin transcript via RNAi (VgRNAi). These data suggest that VgRNAi increases lifespan through the trade-off between reproduction and longevity and animals with combined ovariectomy and VgRNAi, might show additive physiological responses. In this study, we used two injection control groups for the VgRNAi treatment, namely buffer injection or injection with RNAi against a 90kDa hexamerin storage protein (Hex90RNAi). We have combined these manipulations to test lifespans upon: OVX & VgRNAi, OVX & Hex90RNAi, OVX & Buffer, Sham & VgRNAi, Sham & Hex90RNAi, and Sham & Buffer. Ovariectomy and VgRNAi exhibited similar reductions in feeding (~40%) and extensions in lifespan (13-21%) but showed differences in vitellogenin protein levels. This study also observed the effects of reduced reproduction on hexamerin storage proteins. We observed that upon ovariectomy and VgRNAi, hexamerins were increased, emphasizing the importance of protein in insect life extension. When methods to reduce reproduction were combined (OVX VgRNAi), no additive physiological responses were observed, suggesting ovariectomy and VgRNAi each extend lifespan by overlapping or convergent pathways.

Introduction

Reduced reproduction increases lifespan in many animals (Flatt 2011). Direct reduction in reproduction can be accomplished through surgical removal of the ovaries (ovariectomy) in grasshoppers (Hatle *et al*., 2008), mutations in ovarian development of *Drosophila* (Flatt *et al.,* 2008), irradiation (Sgró *et al*., 1999), and surgical ablation of the germ-line stem cells in *C. elegans* (Arantes-Oliveira *et al.,* 2002), to name a few. In each of these manipulations, lifespan was increased over controls, demonstrating that reduction in reproduction can increase lifespan. In spite of how widespread this trade-off is, a mechanism underlying it

has not been identified (Flatt 2011). Previous studies have suggested that the tradeoff between longevity and reproduction is due to a tradeoff in resource allocation (Kirkwood 1977), hormonal regulation (Ghazi 2012) or antioxidant activity (Hatle *et al*. 2008). However, recent studies have shown that these mechanisms may not alter reproductive investment (Judd *et al*., 2011, O'Brien *et al*., 2008, Grandison *et al*., 2009). Hence, physiological mechanisms underlying the trade-off of reproduction with lifespan are of interest.

Different means of reducing reproduction have been shown to increase lifespan and affect the physiology of organisms in distinct ways. These always include dietary restriction as one of the means of reducing reproduction. For example, in grasshoppers, ovariectomy results in 5-fold increases in the reproductive protein vitellogenin and decreases in anti-oxidant activity, however upon dietary restriction, vitellogenin levels and anti-oxidant activities are unchanged (Drewry *et al*., 2011). In *Caenorhabditis elegans*, surgical ablation of germ-line precursor cells result in increased lifespan, yet removal of the entire gonad will not promote longevity (Hsin *et al*., 1999). When germ-line cells are removed in in *C. elegans* and subjected to dietary restriction, lifespan is not increased additively (Crawford *et al*., 2007). In addition, down-regulation of insulin signaling and dietary restriction are shown to extend lifespan in *Drosophila*, but when FOXO transcription factors or insulin-like peptides are knocked down in the insulin-signaling pathway, dietary restriction will extend lifespan on its own (Min *et al*., 2008). These data suggest that when organisms are exposed to dietary restriction in addition to a method to reduce reproduction, each which independently extends lifespan, there are no additive effects on longevity, which suggests they act on the same or convergent life-extending pathways. Alternatively, Drewry et al. (2011) showed that reducing reproduction by ovariectomy and dietary restriction resulted in additive lifespans, proposing that these two life-extending treatments act on separate pathways. On account of these findings, it is unclear if all methods of extending life act on the same or similar physiological pathways. To our knowledge, there have been no studies testing the effects of directly reducing reproduction in two separate ways to determine if there are potential additive effects on life extension.

For this study, we directly reduced reproduction in two ways by focusing on vitellogenin and removal of the ovary. Vitellogenin is mediated by the target of rapamicin (TOR) and stimulated by the insulin signaling (IIS) pathway, in cockroaches and worms respectively (Maestro *et al*., 2009, DiPina *et al*., 2011). This protein, which is essential for egg production and reproduction, also affects an organism's lifespan (Murphy *et al*., 2003, Amdam *et al*., 2004). It is therefore important to look into how decreased vitellogenin, in addition to surgical removal of the ovaries, affects grasshopper physiology and lifespan.

To investigate this trade-off and study the effects of reproduction on aging, we used the Eastern lubber grasshopper, *Romalea microptera.* These animals are easy to rear, have easily determined feeding rates, and are large enough upon which to conduct surgeries. We can directly reduce reproduction by removal of the ovary (Hatle *et al*., 2003) which increases lifespan by ~20%, in old grasshoppers reduces feeding by ~50%, and increases hexameric storage proteins (Hatle *et al*. 2008). Ovariectomy has been shown to double fat body mass and hemolymph volume, indicating an overall increase in storage (Hatle *et al*., 2013). Yet, ovariectomy does not completely remove the grasshopper's ability for investment in reproduction. These animals can still produce the protein vitellogenin, the egg yolk-precursor protein that makes up ~90% of the protein content in the egg. However, with ovariectomy, there is no ovary to sequester the vitellogenin from the hemolymph (Hatle *et al.,* 2001 and Borst *et al.,* 2000). This results in a 5- to 10-fold increase of vitellogenin in the hemolymph (Hatle *et al*., 2008).

As a second, separate means of directly reducing reproduction, we can decrease vitellogenin mRNA by knocking it down using RNA interference (Tokar *et al*., in preparation). Though vitellogenin mRNA is reduced, Tokar et al. (in preparation) found that vitellogenin protein levels are actually increased upon vitellogenin RNAi (VgRNAi). Decreasing reproduction in grasshoppers through VgRNAi results in a doubled fat body mass, nearly halts reproductive development for at least the first clutch of eggs, and lowers ovarian masses by 7-fold (Tokar *et al*., in preparation). Due to the changes in reproductive investment, these data suggest that Vg-RNAi might affect longevity.

Hence, Vg-RNAi has several similar phenotypic effects to ovariectomy. However, are not identical treatments, as previous studies have shown differences in vitellogenin mRNA, vitellogenin protein, and hexamerin protein levels. Ovariectomized animals had 20-fold lower levels of vitellogenin mRNA, 5-10-fold higher levels of vitellogenin protein, and 3- fold higher levels of hexamerin storage protein in the hemolymph (Drewry *et al*., 2011 and Judd *et al.,* 2011), while animals with Vg-RNAi treatments showed, 40-fold lower levels of vitellogenin mRNA, 3-fold higher levels of vitellogenin protein, and no significant differences in hexamerin levels relative to controls (Tokar et al., in preparation) suggesting that these two processes may act through different pathways.

Lipid storage has also been found to be important for an organism's lifespan (Hansen *et al*. 2013, Chapman 1998; Hatle *et al*., 2006, Wang *et al*., 2008). However, in insects it is protein storage that is most important in reproduction. It is unclear what role storage protein levels play in longevity. In the eastern lubber grasshopper as 80% of non-vitellogenin proteins in the hemolymph are hexamerins. In fully fed females, these proteins peak 22 days after molt, and fall during oviposition. The maximum levels of hexamerins will predict the number of eggs laid for that clutch (Hatle *et al*., 2001). *R. microptera* has three hexamerins (Hex-500, Hex-270, Hex-90; Hathaway et al. 2009). Though these hexamerins may affect lifespan, they have been shown to not affect reproduction directly. It is because of this, we chose to knockdown the 90kDa hexamerin (Hex-90) as a control for RNAi mechanistic effects.

This study addresses whether Vg-RNAi increases lifespan. Feeding rates, reproductive output, and quantities of vitellogenin and hexamerin in hemolymph aided in interpretations of effects of ovariectomy and RNAi on the animal. Further, it was tested whether ovariectomy and Vg-RNAi treatment combined within the same individuals have additive effects on lifespan which would suggest that OVX and Vg-RNAi act through separate mechanisms.

Methods

Animal rearing

Juvenile eastern lubber grasshoppers (*Romalea microptera*) were obtained from Miami, Florida, USA, were raised communally, and fed an ad libitum diet of Romaine lettuce (Hatle et al., 2008). Females were separated upon adult molt, individually placed into 500 cm^3 containers, and reared in an environmental chamber (14L:10D, 35°C:27°C, 50%RH) (Hatle et al., 2001). Adults were fed an ad libitum diet of Romaine lettuce. Feeding rates were determined every 7 days by drying the food left uneaten and converting dry mass of lettuce to wet mass using known amounts of wet lettuce (Hatle et al., 2006).

Surgical and injection assignments

Animals were serially assigned into control operated (Sham) or ovariectomized (OVX) surgical groups. Surgeries were conducted one to two days after adult molt (Hatle et al., 2003). Within the surgical groups, individuals were serially assigned into three injection groups: RNAi treatment for vitellogenin (VgRNAi), RNAi treatment for a 90kD hexamerin storage protein (Hex90RNAi), or the carrier Tris buffer (Buffer).

Synthesis and injection of dsRNA

Double-stranded RNA (dsRNA) for RNAi was synthesized according to the instructions of Ambion's MEGAscript® RNAi Kit (Life Technologies, Grand Island, NY). The DNA template used was from fat body cDNA of a vitellogenic female grasshopper (Tokar *et al*., in preparation). Template or primer concentrations for PCR were reduced until pure product was obtained, as determined by melt curves and gel electrophoresis. dsRNA product was analyzed by agarose gel to ensure the product was the predicted size, and purity was tested by spectrophotometry.

Four to five days after adult molt, individuals were injected intra-abdominally with 5 µg of dsRNA in 30 µl buffer, or only 30 µl of Tris buffer, according to injection assignments (Tokar *et al*., in preparation). This led to a fully factorial 2 x 3 design of Sham VgRNAi, Sham Hex90RNAi, Sham Buffer, OVX VgRNAi, OVX Hex90RNAi, and OVX Buffer (Mean n per group = 25.17, Range= 25-26).

Oviposition, collection of hemolymph, and survivorship

Approximately 30 days after adult molt all grasshoppers were placed on damp sand two times a week to allow for oviposition (Hatle et al., 2001). When deposition of eggs occurred, the eggs were counted, measured, and age at ovipositon was recorded.

Starting at about 30 days after adult molt, monthly hemolymph samples were taken from approximately one-half of the animals in the study (Hatle et al., 2006). Each sample of 5 µL was placed in 250 µL of phosphate buffered saline and stored at -80°C. These were used for vitellogenin measurement using an ELISA (Borst et al., 2000) and hexamerin measurement using native PAGE.

Deaths were recorded daily to determine survivorship. The experiment was terminated when animals were at a median age of 213 days and the control Sham Buffer group had a survivorship of 4%.

Statistics

Data was analyzed using seven two-way MANOVAs with time as an independent variable. The first MANOVA tested the effects of surgery and injection on amounts of food eaten. The second MANOVA tested the effects of surgery and injection on body masses. The third MANOVA tested the effects of injection on the age of the animal in which clutches were laid, number of eggs produced per clutch, and lengths of eggs. The fourth MANOVA tested surgery and injection effects on vitellogenin levels in the hemolymph. The fifth, sixth, and seventh MANOVA tested the effects of surgery and injection on levels of three hexamerins in the hemolymph. Mortality rates were tested using Cox proportional hazards models (Allison 2010).

Results

Food eaten - MANOVA showed a strong effect of age (Pillai's Trace $F_{14,31}$=11.70, P<0.0001), no interaction of age and surgery ($F_{14,31}$=1.85, P=0.075), and no interaction of age and injection ($F_{28,64}$=1.29, P=0.20). Ovariectomy resulted in a significant decrease in consumption of Romaine lettuce after grasshoppers reached a median age of 56 days, continuing through median age of 105 days (all

P<0.04). Injection showed a reduction in lettuce consumption at 42 days ($F_{2,5}$=3.53, P=0.038), however, at all other time points the amount of lettuce was not affected by injection (all P>0.07). Overall, decreased reproduction (OVX Buffer, OVX Hex90RNAi, OVX VgRNAi, and Sham VgRNAi) resulted in reductions in feeding rates after day 63, regardless of treatment method (all P<0.025). Feeding rates tend to decrease prior to oviposition. In Sham Buffer and Sham Hex90RNAi animals, first oviposition occurred at 40.3 ± 5.8 days (mean ± SE), likely explaining the general decrease in feeding for all treatments around day 42 (Fig. 1). Feeding data was collected until day 105 where sample sizes were no longer sufficient to conduct statistical analyses.

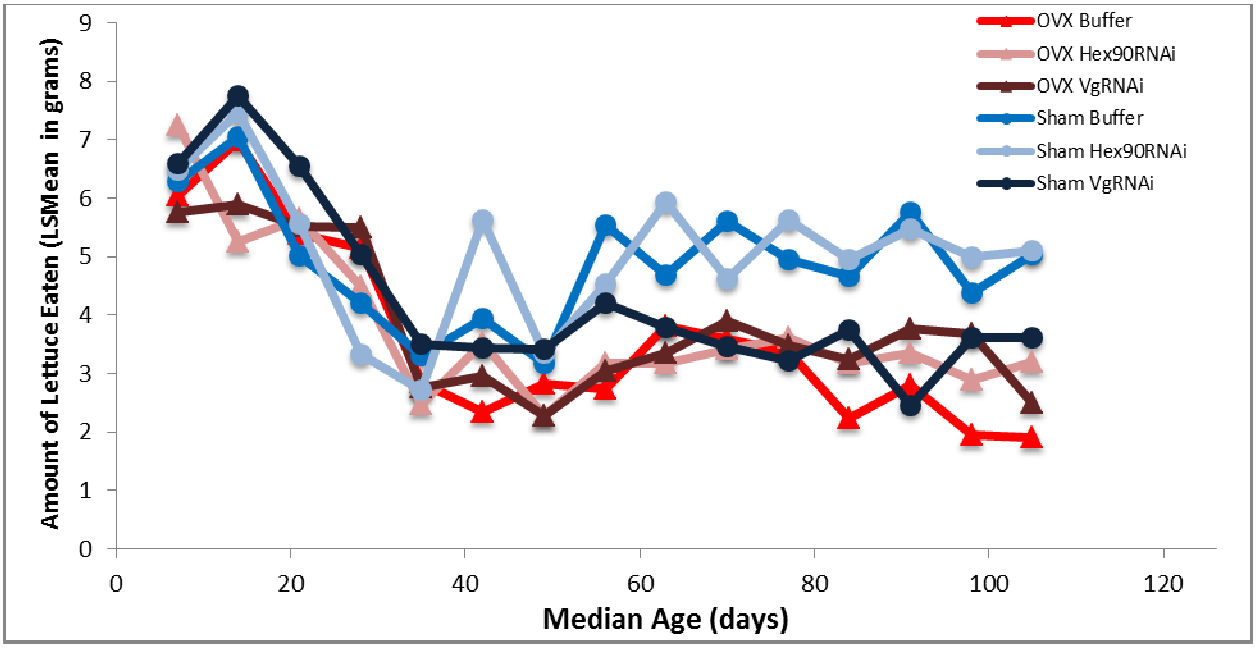


Figure 1. Mean amounts of Romaine lettuce eaten daily by each treatment group of adult lubber grasshoppers. The Sham VgRNAi group and all three ovariectomized groups demonstrated decreases in feeding rates relative to Sham Buffer (error bars omitted for clarity).

Body masses - There was a strong effect of age (Pillai's Trace F*17,56*=73.36, P<0.0001), and an interaction of age and surgery (F*17,56*=3.29, P=0.0004), yet no overall interaction of age and injection (F*34,114*=1.41, P=0.09). Body masses for all treatments increased rapidly until median age of 24 day, and then increased slowly until median age of 42 days, which is just following the oviposition of clutch 1 (Fig. 2). Starting at 49 days, non-reproductive animals exhibited steady increases in body mass relative to fully reproductive individuals (all P<0.0005). Ovariectomized animals exhibited steady increases in body masses relative to

sham operated individuals (all P<0.05), and Sham VgRNAi individuals began to show increases in body masses at a median age of 63 days, then maintained average masses (9.9 g ± 0.36), approximately equal to ovariectomized animals (9.3 g ± 0.34).

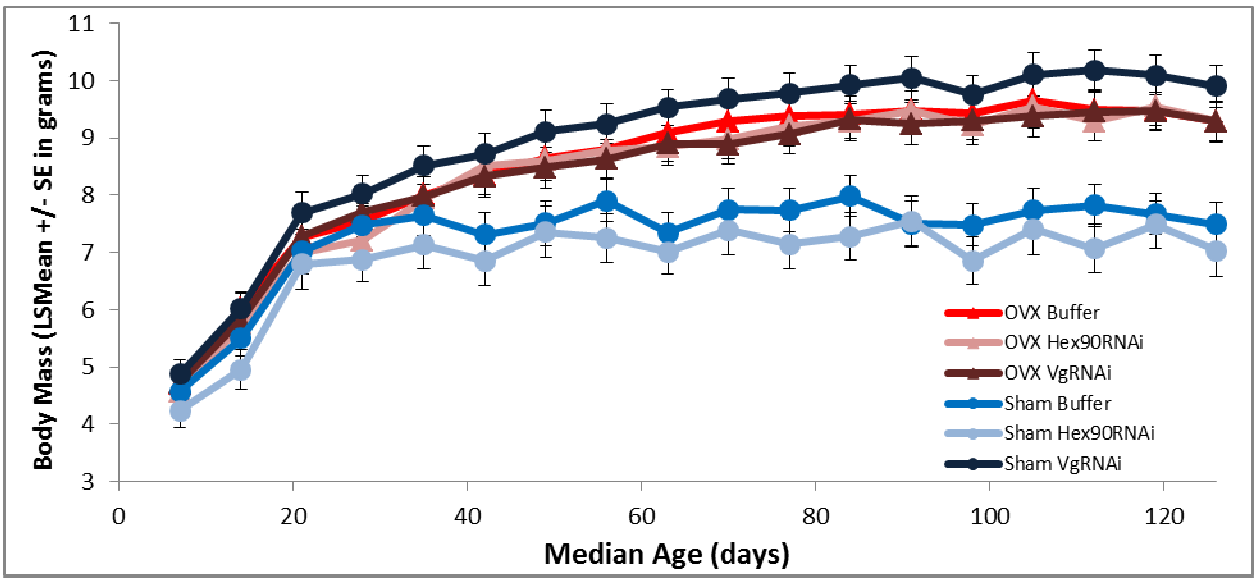


Figure 2. Ovariectomized grasshoppers had higher body masses than Sham operated groups over their lifespan (all P<0.05). Sham VgRNAi individuals showed similar body masses to OVX groups after a median age of 63 days. Hence, reduced reproduction increased body mass.

Reproductive output - All individuals were given access to sand for deposition of eggs. Ovariectomy resulted in no deposition of eggs. Among sham-operated groups, VgRNAi greatly reduced reproductive output. First, VgRNAi reduced the number of clutches in comparison to other sham-operated groups (Mean clutches: Sham Buffer = 5.60 ± 0.4, Sham Hex90RNA = 4.52 ± 0.6, Sham VgRNAi = 0.44 ± 0.2). Because of this, MANOVA was run only through clutch 2, and included 3 out of 25 Sham VgRNAi individuals that laid 2 clutches. For all clutch parameters (age at clutch, number of eggs, and length of eggs), there was a strong effect of age (Pillai's Trace $F_{5,40}$=489.90, P<0.0001) and an interaction of age and injection ($F_{10,82}$=7.56, P<0.0001). Sham Buffer and Sham Hex90RNAi exhibited no difference in age at the laying of each clutch (all P>0.96), number of eggs (all P>0.76) (Fig. 3), or length of eggs in clutches 1 and 2 (all P>0.23). Sham VgRNAi animals laid eggs at much older ages (all P<0.0001), produced fewer eggs (all P<0.0001), and had smaller eggs than Sham Buffer and Sham Hex90RNAi (all P<0.01).

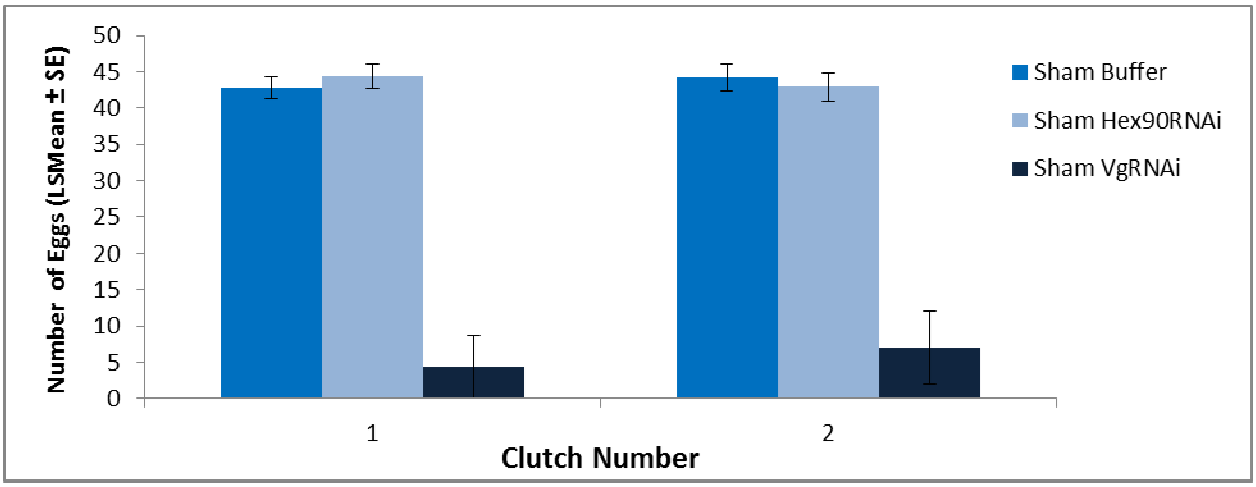


Figure 3. Egg numbers per clutch in Sham operated grasshoppers. Sham VgRNAi individuals laid fewer eggs in clutch 1 and 2 than Sham Buffer and Sham Hex90RNAi individuals (all P<0.0001).

Life spans - Surviving animals were terminated at a median age of 213 days (209-222 days). Median life spans were: OVX Buffer = 169.5 days, OVX Hex90RNAi = 150.5 days, OVX VgRNAi = 160.5 days, Sham Buffer = 147.5 days, Sham Hex90RNAi = 134.5 days, and Sham VgRNAi = 169.5 days (Fig. 4). COX proportional hazards models revealed that ovariectomy ($\chi 2$ =5.17, P = 0.016) and injection ($\chi 2$ = 8.29, P = 0.02) each increased survivorship. There was an interaction of surgery and injection ($\chi 2$ = 12.10, P = 0.002) implying that the treatments were not additive.

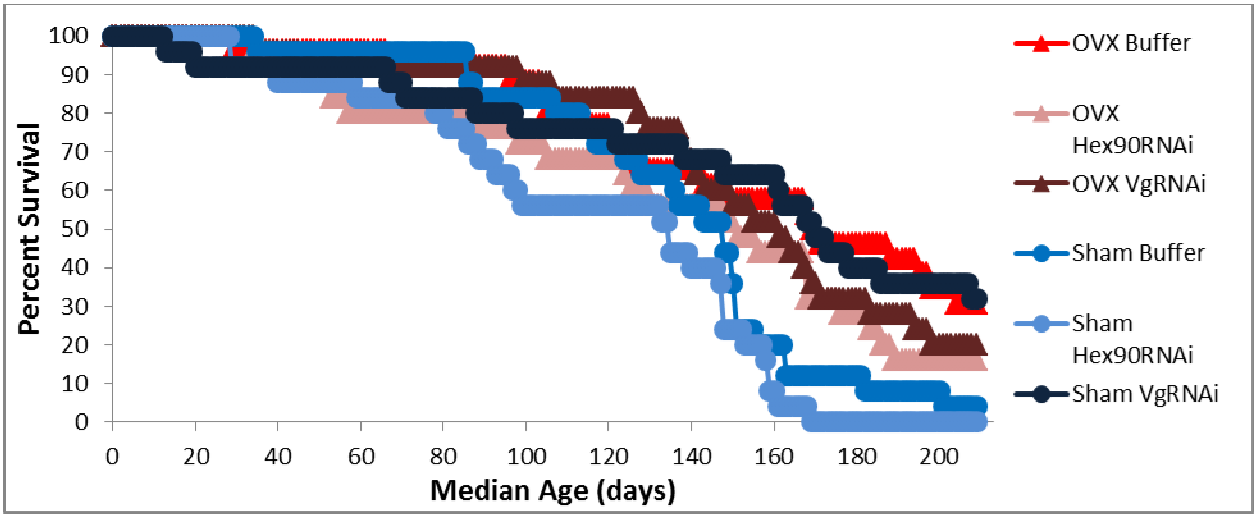


Figure 4. Grasshoppers with reduced reproduction showed higher survivorship compared to fully reproductive groups (OVX: P=0.016, VgRNAi: P=0.02). However, combined ovariectomy and VgRNAi injection groups (OVX VgRNAi) did not result in additive survivorship.

Vitellogenin levels in the hemolymph - MANOVA shows no effect of age on vitellogenin (Pillai's Trace $F_{3,46}$=1.12, P=0.35). There was also no interaction of age and surgery ($F_{3,46}$=1.08, P=0.36), and no interaction of age and injection ($F_{6,94}$=0.97, P=0.45) . In contrast, ovariectomy resulted in a 5- to 12-fold increase in vitellogenin levels in the hemolymph through 120 days after adult molt (all P<0.001); OVX VgRNAi groups demonstrated decreases in vitellogenin levels (Mean=42.4 ± 38.2) relative to OVX Buffer (Mean=278.1 ± 36.4) and OVX Hex90RNAi (Mean=258.8 ± 40.2) (all P<0.05 starting at day 30). However, Sham VgRNAi groups showed no differences in vitellogenin levels relative to other sham groups (all P>0.92) (Fig. 5). Overall, non-reproductive animals showed higher vitellogenin levels in the hemolymph relative to reproductive groups (all P<0.05).

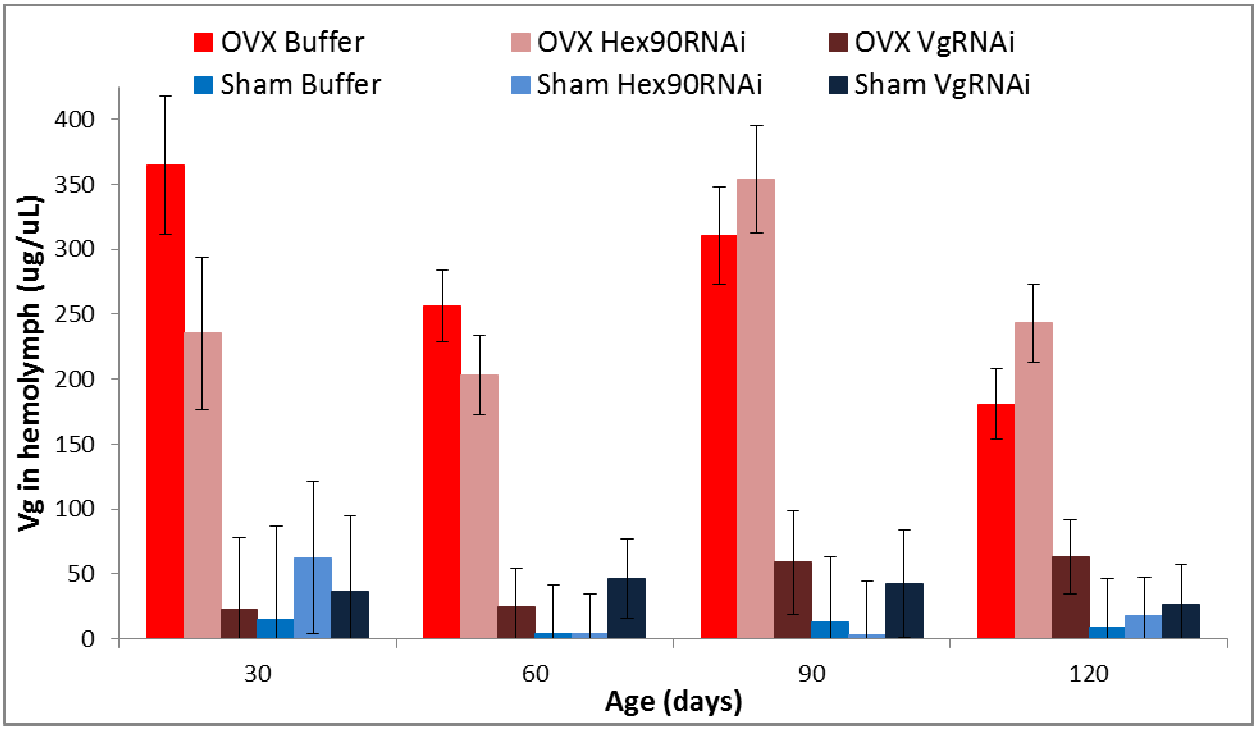


Figure 5. Ovariectomy resulted in an increase in vitellogenin for OVX Buffer and OVX Hex90RNAi groups relative to the OVX VgRNAi treatment (all P <0.001). There were no differences in vitellogenin for the sham operated groups (all P>0.92).

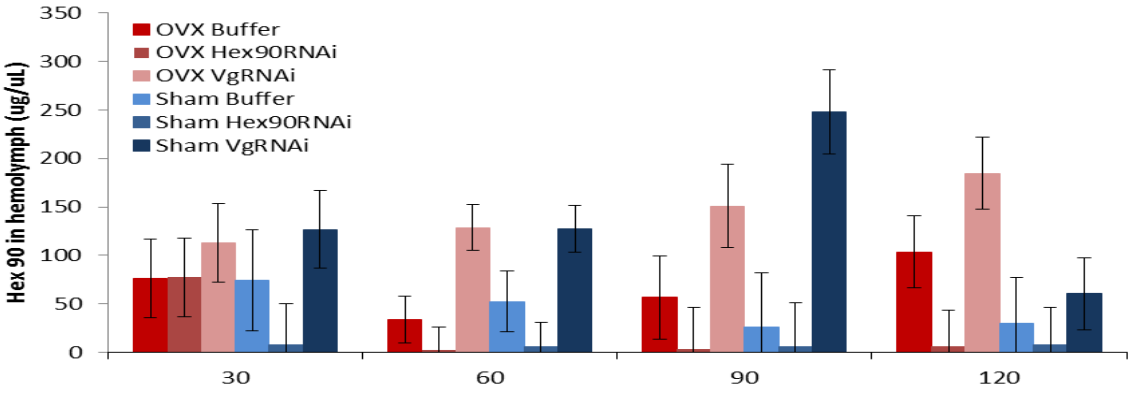
Hexamerin levels in the hemolymph - All hexamerins increased upon ovariectomy at 120 days. VgRNAi treatment resulted in increases in hexamerins 90 and 270 after 60 days but did not demonstrate increases in hexamerin-500. The three MANOVAs on hexamerins showed no interaction effects on age, age and

surgery, and age and injection (Age: Hex500: F*3,42*=1.50, P=0.23; Hex270: F*3,47*=0.70, P=0.56; Hex90: F*3,46*=0.64, P=0.60, Age and surgery: Hex500: F*3,42*=2.40 P=0.08, Hex270: F*3,47*=0.57, P=0.64, Hex90: F*3,46*=1.23, P=0.31, Age and injection: Hex500: F*6,86*=1.38 P=0.23, Hex270: F*6,96*=2.01, P=0.07, Hex90: F*6,96*=0.87, P=0.52).

Hexamerin-90 was 3-fold greater upon ovariectomy at 120 days (P=0.05) and increased with VgRNAi at 60 days (2-fold, P=0.01) and 90 days (4.5-fold, P=0.004). Hex90RNAi resulted in decreased hexamerin-90 in the hemolymph after day 30. Effects were significant to VgRNAi (after day 30 all P<0.01) but not Buffer treated individuals (after day 30 all P>0.31).

Hexamerin-270 increased 3-fold upon ovariectomy at 120 days (P=0.03) and increased 3.5-fold with VgRNAi at 60 days (P=0.003).

Hexamerin-500 increased upon ovariectomy at 90 (3-fold; P=0.01) and 120 days (2-fold; P=0.01) but did not change due to Vg-RNAi (Fig. 6).



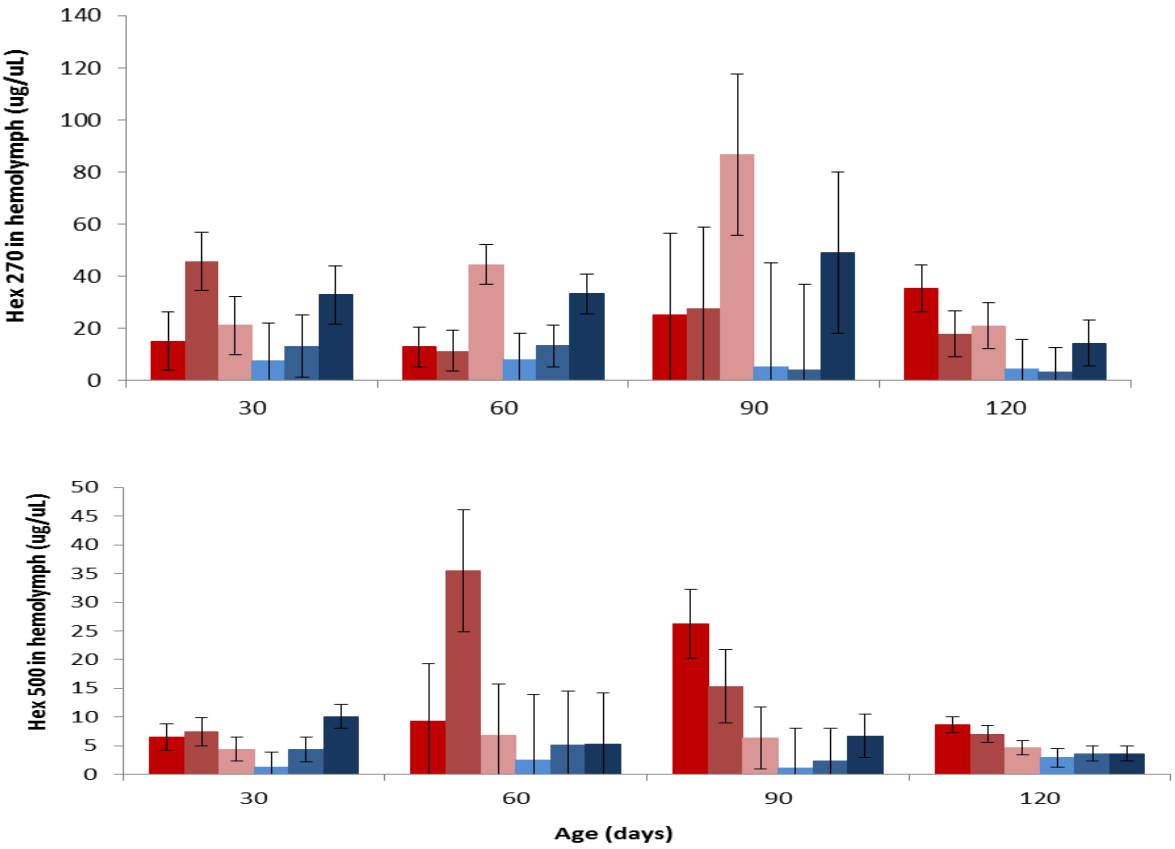


Figure 6. All hexamerins increased upon ovariectomy after 120 days (Hex-90: P=0.05, Hex-270: P=0.03, Hex-500: P=0.01). VgRNAi treatment resulted in increases in hexamerin 90 (P<0.01), and 270 (P=0.003) but not in 500.

Discussion

The basis of this study was to determine if reduction of vitellogenin mRNA extends lifespan and if ovariectomy and vitellogenin mRNA knockdown results in additive effects in lifespan extension. We hypothesized that the grasshopper might exhibit additive effects on lifespan with a combination of ovariectomy and VgRNAi (OVX VgRNAi), implying that these two distinct routes of reducing reproduction will extend lifespan through different pathways in the lubber grasshopper. Our data show that VgRNAi treatments and ovariectomy each result in similar increases in lifespan, body mass, and storage protein levels, and a similar decrease in feeding rate. Alternatively, this study shows vitellogenin protein levels in the hemolymph were much greater in ovariectomized relative to VgRNAi treated animals. These two

methods to reduce reproduction result in similar physiological responses yet they are not completely identical.

Feeding rates similarly decrease upon ovariectomy and VgRNAi

Ovariectomized as well as Sham VgRNAi animals exhibited similar decreases in feeding rates relative to fully reproductive controls (Sham Buffer, Sham Hex90RNAi). Animals with a combined ovariectomy and VgRNAi treatment (OVX VgRNAi) maintained the same feeding levels as the other reduced reproduction groups. Drewry et al. (2011) showed that ovariectomy reduces feeding (~40%) and proposed that other ways to reduce reproduction (VgRNAi) may as well. It is therefore useful to determine feeding rates alongside any manipulation in reproduction. Grasshoppers offer the ability to easily determine feeding rates, whereas commonly used genetic models are much more difficult due to their small size (Min *et al*. 2007, Piper *et al*. 2007). Since each method to reduce reproduction independently resulted in similar feeding responses, it is likely that ovariectomy and VgRNAi reduce feeding by the same mechanism, explaining why we do not see an additive increase in feeding in OVX VgRNAi animals. Several insects including *Drosophilia*, locusts, and honey bees have demonstrated that Neuropeptide F (NPF) is an important regulator of feeding and forging behavior (Wielendaele *et al*., 2013). With reductions in NPF, feeding behavior decreases. It is possible that in this study both ovariectomy and VgRNAi resulted in decreases in NPF, consequently leading to reductions in feeding for both treatments.

VgRNAi reduces, but does not eliminate reproductive output

VgRNAi greatly reduced reproduction late in life. A single injection of double-stranded RNA against vitellogenin approximately 5 days after adult molt resulted in significant decreases in all reproductive parameters. Even though 24% of Sham VgRNAi animals deposited eggs, the egg number was dramatically decreased and the clutch duration was dramatically increased relative to the other Sham-operated groups. Ultimately VgRNAi did not completely eliminate all reproductive output but our data showed large reductions throughout the entirety of the grasshopper's lifespan.

Lifespan is increased upon VgRNAi

Grasshopper lifespan was increased by VgRNAi. Reproduction is widely thought to have a costly effect on energy and maintenance, ultimately shortening lifespans (Hansen *et al*., 2013). VgRNAi reduces reproduction by decreasing mRNA levels of vitellogenin and halting ovarian development (Tokar *et al*., in preparation). We saw that Sham VgRNAi animals lived 13-21% longer than either of the Sham operated controls, and similar to the degree to which ovariectomy extends lifespan. When ovariectomy and VgRNAi treatments were combined, it resulted in an intermediate survival rate between OVX Buffer and Sham Buffer treated animals. Vitellogenin knockdown may generally increase lifespan in non-social insects, and it may act on the same pathway of life extension as ovariectomy.

Ovariectomy and VgRNAi differ in ecdysteroid and vitellogenin protein levels

These two treatments (i.e., ovariectomy and VgRNAi) are distinct. In the grasshopper, reproductive hormonal signaling is maintained despite the removal of the ovary or VgRNAi treatment, as the main gonadotropin (Juvenile Hormone) is made in the corpora allata (in the head) (Hatle *et al*., 2003). This is unlike commonly used genetic models in which vitellogenin production is halted with removal of the ovary (Chapman 1998 and Hatle *et al*., 2003). Upon VgRNAi, juvenile hormone (Tokar and Hatle, unpublished) acts on the fat body still producing vitellogenin mRNA, but RNAi effects break apart this transcript resulting in decreases in vitellogenin mRNA abundance. Therefore, the Sham-VgRNAi grasshoppers had decreases in vitellogenin mRNA, but still possessed their ovary. Those with ovariectomy and injection controls (OVX Buffer, OVX Hex90RNAi) maintained high levels of vitellogenin mRNA but did not possesses an ovary. And finally, those with ovariectomy and VgRNAi treatments (OVX VgRNAi) had decreased vitellogenin transcript and no ovary. In all ovariectomized grasshoppers, ovariectomy resulted in neither ovarian development nor deposition of eggs. The ovary is the only significant source of ecdysteroids in the hemolymph of adult female grasshoppers. They are present in high levels two weeks before oviposition, but do not affect vitellogenesis. Nonetheless, they can be markers for reproductive development (Hatle *et al*., 2003). In a similar study, upon VgRNAi, ecdysteroid levels were no different from buffer injected or Hex90RNAi treatments (Tokar *et al*. in preparation) but also shows that ovariectomy results in low ecdysteroid levels. This suggests there was maintenance of some ovarian

activity in Sham VgRNAi animals. Additionally, ovariectomy results in increased vitellogenin protein levels while all animals given VgRNAi treatments showed low levels of vitellogenin protein. These physiological responses emphasize that ovariectomized and VgRNAi treated animals are not identical treatments.

Vitellogenin RNAi injection reduced vitellogenin protein in ovariectomized animals but not in sham-operated females. In Drewry et al. (2011), ovariectomy showed large increases in vitellogenin relative to Sham operated groups. With removal of the ovary, vitellogenin is still produced but cannot be sequestered into the oocytes, explaining the increases in its levels in the hemolymph. With the combined treatments of ovariectomy and VgRNAi (OVX VgRNAi), vitellogenin protein levels were reduced 6-fold compared to other ovariectomy treatments, suggesting a successful vitellogenin knockdown. It is important to note that the vitellogenin protein levels in OVX VgRNAi and Sham VgRNAi are not significantly different. It is possible that VgRNAi may exhibit an incomplete knockdown effect and can only reduce vitellogenin protein levels when they would be very high, as upon ovariectomy. Tokar et al. (in preparation) show that vitellogenin mRNA levels in the fat body were decreased 35-fold upon VgRNAi compared to controls. They also found increased hemolymph vitellogenin levels with VgRNAi treatment; however this study was only conducted at younger ages (33 days old). Levels of mRNA and levels of proteins often correlate poorly. For example, studies featuring yeast and cancer cells have shown very little association between mRNA and protein (Maier *et al*., 2009). It is possible that pre-and post-transcriptional modulators, as well as changes in protein half-lives, contribute to the differences seen between vitellogenin mRNA levels from Tokar et al. (in preparation) and vitellogenin protein levels from this study. Independent of the lack of correlation between mRNA and protein levels, VgRNAi was successful in nearly halting reproductive development in sham-operated animals and reducing vitellogenin protein levels in ovariectomized grasshoppers.

Vitellogenin may be mediated by converging, life-extending, pathways

The trade-off between reproduction and longevity is commonly seen (Flatt 2011). In some species, vitellogenin levels are negatively correlated with longevity. For example, *C. elegans* that have reduced activity of vitellogenin encoding genes *vit-2* and *vit-5* will exhibit lengthened lifespan (Murphy *et al*.,

2003). Vitellogenesis is regulated by juvenile hormone (JH) in non-dipteran insects, and JH is in turn regulated by nutritional signals that are controlled through the target of rapamycin (TOR) pathway (Maestro *et al*., 2009). Several studies have shown there to be transcription factors (DAF-16/FOXO, HSF-1) that are common in two separate important aging pathways, TOR and insulin-like signaling (IIS) (Seo *et al*., 2013). Vitellogenin is also produced upon stimulation of insulin-like signaling mechanisms, and this pathway may be important in reallocating resources from somatic preservation to reproduction (DePina *et al*., 2011). It is possible that in grasshoppers, vitellogenin is mediated by both the TOR and the IIS pathway independently which in turn affects longevity.

Studies featuring the honey bee (*Apis mellifera)* show vitellogenin having the reverse effect, namely a positive correlation to lifespan, which also demonstrates that vitellogenin levels play a large role in the processes of aging (Amdam *et al*., 2004). Humans also have proteins that are members of the vitellogenin family, including microsomal transfer protein (MTP) and apolipoprotein B (APOB) which both influence lipoprotein profiles and metabolism (Brandt *et al*., 2005). Hence it may be beneficial to look at vitellogenin, vitellogenin gene families, and the IIS pathway in other species to determine how these affect lifespan and aging. From our study we can see that VgRNAi affects vitellogenin levels and in turn results in changes in reproduction, storage, and lifespan. It has been found in it can be energetically costly to produce eggs (Yanagi *et al*., 2003, DeLoof 2011). VgRNAi prevents egg growth and increases longevity, falling in line with the hypothesis that the amount of effort and energy expended in reproduction may be inversely proportional to the animal's lifespan.

Protein storage is increased upon VgRNAi

Our study shows that all three hexameric storage proteins in the hemolymph were increased upon ovariectomy late in life and were similarly increased upon VgRNAi treatment with the exception of hexamerin-500. In animals, reproduction and longevity are often linked with storage. Lipid storage is often increased when reproduction is reduced and survival is increased (Hansen *et al*., 2013, Rion *et al*., 2007). Studies have shown that many organisms including *C. elegans*, and mice exhibit this relationship between reproduction, fat storage, and survival (Hansen *et al*. 2013). In addition to the ability to store

lipids, insects (and crustaceans) are able to store amino acids in hexamerin proteins (Tang *et al*., 2010). Hexamerins store these amino acids to be used in metamorphosis, reproduction, and development. These proteins are broken down when food is scarce or amino acid demand is high (Wheeler *et al*., 1995). Long lived insects will show increased storage of carbohydrates and lipids (e.g., Judd et al. 2011, Tartar *et al*., 2001), but it is proteins that are the limited nutrient for development (Chapman 1998; Hatle *et al*., 2006). Grandison et al. (2009) has demonstrated that the increased intake of calories does not affect lifespan, however it is the abundance of amino acids that does and in particular they find that high levels of the amino acid methionine while on a restricted diet is essential for lifespan extension with full reproduction. We hypothesize that any reduction in reproduction, regardless of method, may increase hexamerins, and ultimately methionine, in the hemolymph. The 270KDa hexamerin is methionine rich and made up of approximately 4% methionine (Hathaway *et al*., 2009) and according to our study this hexamerin is increased (among others) with ovariectomy and VgRNAi. If methionine storage is increased under reductions in reproduction, this may be one contributor to the extension of the organism's lifespan. Judd *et al*. (2011) demonstrate that although the grasshopper increases in body mass during ovariectomy and there are increases in somatic storage, there is no increased allocation of nutrients to somatic tissues. While we did not measure allocation of nutrients to storage, our data also shows increases in body mass upon ovariectomy but additionally shows increases in body mass upon VgRNAi. OVX VgRNAi treatments did not show larger body masses than any other reduced reproductive group, again suggesting that the mechanisms prolonging life may be through the same pathways.

In addition to reproduction, hexamerins are important in organism survival. The insect will use these storage proteins for development and somatic maintenance. In our study, organisms with Hex90RNAi injection treatments had decreased lifespans relative to the other animals in their surgical groups. Hex90RNAi was used to control for the general effects of the mechanisms behind RNAi. It is possible that the reduction in lifespan was due to the RNAi mechanisms themselves. In a study conducted by Alic et al. (2012), they suggest that RNAi may have sequence-independent effects on lifespan. If RNAi indeed was behind the shortening of lifespans, this further demonstrates the powerful, life extending effect of

VgRNAi in grasshoppers. Alternatively, the most abundant hexamerin found in *R. microptera* (Hex-90) was knocked down, which may have ultimately reduced the organism's ability to use these amino acids for survival.

Conclusions

When methods to reduce reproduction were combined (OVX VgRNAi), no additive physiological responses were observed, suggesting ovariectomy and VgRNAi each extend lifespan by overlapping or convergent pathways. It has been documented that vitellogenin plays a role in the TOR and IIS longevity pathways. Therefore, it would be acceptable to suggest that VgRNAi may affect TOR and IIS as well. While TOR or IIS signaling has not been tested with ovariectomy, we propose that ovariectomy may affect these pathways as well. These two treatments may affect different transcription factors that ultimately converge on these pathways.

As these two methods of reducing reproduction resulted in similar effects in the organism and may act upon the same life-extending pathways, it appears that it would be unnecessary to combine other methods of reducing reproduction (eg. dietary restriction, or removing the corpora allata) to observe longevity. Future endeavors might be best focused on observing effects of ovariectomy on the IIS or TOR transcription factors using next-generation transcriptome sequencing.

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Vita

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