PROXIMATE MECHANISMS UNDERLYING PLASTICITY IN LIFE HISTORY UNDER VARIABLE DIETARY CONDITIONS IN THE MILKWEED BUG, *ONCOPELTUS FASCIATUS*

by

ASHLEY EMILY DUXBURY

(Under the Direction of Patricia J. Moore)

ABSTRACT

Oncopeltus fasciatus males fed a diet of either milkweed seeds, the ancestral state, or sunflower seeds, the adapted state, have different reactions to their diet. Milkweed-fed males live a shorter life, while investing more in reproduction, while sunflower-fed males live longer, but at the expense of their fertility. To examine this trade-off further, I studied the fertility and fecundity of males on each diet as well as looked at the genes that were differentially expressed under each dietary condition. Males fed milkweed-seeds have more sperm than sunflower males at an older age, but not higher quality sperm, and older milkweed fed males are more likely to fertilize eggs than old sunflower-fed males. To determine if the change in fecundity and amount of sperm was due to a genetic factor, a transcriptome was completed on the testes of *O. fasciatus* males. The distal tip of the testis was separated from the proximal rest of the testis to try and separate the germline stem cell maintenance and activation genes from spermatogenesis genes. It was found that there were indeed differences in the distal tip and rest of the testes within as well as between the diets. Overall, there are candidates for further

studies into the mechanisms behind life history plasticity that may lead to an answer as to what causes this trade-off between reproduction and longevity.

INDEX WORDS: life history, transcriptome, diet, fecundity, fertility, spermatogenesis, germline stem cells

PROXIMATE MECHANISMS UNDERLYING PLASTICITY IN LIFE HISTORY UNDER VARIABLE DIETARY CONDITIONS IN THE MILKWEED BUG, *ONCOPELTUS FASCIATUS*

by

ASHLEY EMILY DUXBURY

B.S., North Carolina State University, 2012

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2017

PROXIMATE MECHANISMS UNDERLYING PLASTICITY IN LIFE HISTORY UNDER VARIABLE DIETARY CONDITIONS IN THE MILKWEED BUG, *ONCOPELTUS FASCIATUS*

by

ASHLEY EMILY DUXBURY

Major Professor:	Patricia J. Moore
Committee:	Gaelen R. Burke
	Ken Ross

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2017

DEDICATION

To Ms. LeGacy and Ms. Wise. Without you two inspiring women, I would not have chosen to go into the field of science. You knew I had it in me before I did.

ACKNOWLEDGEMENTS

This dissertation becomes a reality with the kind of support and help of many individuals. I would like to extend my sincere thanks to all of them.

My advisor – Dr. Patricia J. Moore for her expertise and knowledge throughout these studies.

My committee members - Drs. Gaelen Burke, and Ken Ross, for sharing your knowledge and supervision of my dissertation.

My family – you have encouraged me throughout this whole process, and I am sure you will be happy when I get a “big kid” job.

Allison – you have been there when I have needed a friend, and you have never left. Thank you.

Morgan and Ruby – you two have always made sure I’m taken care of when I need to be.

Tracey – you have kept me sane and helped me learn about myself these past 5 years.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Life history is built upon trade-offs in which one trait affects one or many others. Reproduction is a constraint that can affect the longevity of an organism. Longevity can be shortened when an organism allocates more resources to reproduction due to a high-quality diet, or longevity can be lengthened with a low-quality diet when an organism allocates less resources to reproduction; the organism is wanting to survive, not to reproduce. Nutrition plays the biggest role in this trade off (Grandison et al. 2009) versus dietary restriction. How does this nutrition act upon the signals that regulate germline stem cells and spermatogenesis to reflect this life history trade-off?

The question has been studied immensely in *Drosophila melanogaster,* mice and *Caenorhabditis elegans*, however, many other insects, or organisms, have not been studied. This is a look into the species of *Oncopeltus fasciatus* where there have been a few studies on the life history and more specifically this reproduction-longevity trade-off; but not as much of an in-depth look as in *D. melanogaster* and *C. elegans*. These organisms have laid the groundwork upon which our studies have been completed.

BASIC BIOLOGY

Oncopeltus fasciatus is in the order Hemiptera and family Lygaeidae, or seed bugs, and live up to their name and eat seeds out of the seed pod of milkweed plants. The species is considered “milkweed-specific” because it is restricted to milkweeds and other plants in the

subfamily *Asclepiadaceae*. These plants, and therefore *Oncopeltus* genus, can be found throughout the North and Central Americas. When found in the wild, they can be found on buildings or on telephone poles and in large groups because of their gregarious nature. The mouthparts can pierce the seedpods of the milkweed plants, which is why the organisms will find a plant with a good number of pods to lay eggs on so that the nymphs have something to eat when hatched. The females usually orient themselves toward the milkweed plants as an oviposition site, through contact chemoreceptors on their antennae (Feir 1974).

O. fasciatus have piercing-sucking mouthparts, often called a rostrum or beak, and the older they get, the thicker the seed pod they can get through, so the organisms can eat multiple parts of the seed pod as they get older. They digest their food externally by secreting the digestive enzymes, amylase, invertase, lipase, and protease, that dissolve the food and then the food is sucked back up through their rostrum to absorb the nutrients (Feir 1974).

These insects are used in the laboratory because of their ease of use and ability to rear in large numbers. In order to sustain large populations required for experiments, *O. fasciatus* laboratory populations have been selected to eat sunflower seeds instead of milkweed seeds in the laboratory. Other diets have also been tested, such as pumpkin, cashew, watermelon. At first, *O.* fasciatus was used for insect physiology studies and recently, molecular techniques have been worked out in the organism. The females lay eggs in clutches of 10 to more than 50 and the eggs hatch in about a week (Feir 1974). Eggs mature to adults through 5 nymphal instars and complete the full transition in 30 days, and the adults can live 4 weeks or longer, depending on their diet. In the lab, these organisms can live in large colonies with water, food, and a place to lay their eggs, usually cotton wool. Our *O. fasciatus* like to have hiding places as

well, we use toilet paper rolls and egg cartons. We keep the organisms in an incubator at 26°C and at 18L:6D. When kept at these conditions, the males become sexually mature between 2 to 3 days and the females become sexually mature between 5 and 12 days after eclosion (Carolina Biological Supply 2008). There is sexual dimorphism, and the females have one stripe and 2 spots on the abdomen and the males have two stripes on the abdomen.

Because of the nature of what the organisms eat, *Oncopeltus* sequester toxic cardenolides and have an aposematic coloration to ward off predators. When an organism eats this insect, it can lead to poisoning symptoms, such as vomiting. These toxic cardenolides, or cardiac glycosides, inhibit the function of intracellular sodium pumps by binding to the alpha subunit of the sodium and potassium ATPase and are highly toxic. In *O. fasciatus*, the cardiac glycosides are sequestered in a double layered epidermis, and are released if the bug is squeezed (Burdfield-Steel and Shuker 2014). This would make it toxic for these organisms to be eaten by any other organism, and therefore they have aposematic coloration of red-orange and black.

These bugs are within the order Hemiptera, suborder Heteroptera, and family Lygaeidae. Lygaeidae *sensu stricto* is traditionally polyphyletic, and are typically considered in the super-family Lygaeiodea and the sister taxa would be Coreoidea and Pyrrhocoroidea. The phylogeny is poorly resolved and more work is clearly necessary (Burdfield-Steel and Shuker 2014). When comparing *O. fasciatus* to *D. melanogaster*, in which most life history studies have been completed, *O. fasciatus* is again used as part of our Evolution and Development model, and this is in part because of its ease of use, but also because it is thought to be part of the group that is part of the closest extant relative to the Holometabola, or the group *D.*

melanogaster is included in, and evolved about 373 million years ago, where the Diptera evolved about 150 million years ago (Misof et al. 2014). This difference can allow us to possibly see if there is a difference in the ways in which these two organisms control germline stem cell maintenance and spermatogenesis.

GERMLINE STEM CELLS (GSCS) AND SPERMATOGENESIS

What are GSCs and how do they become sperm?

In adults, there are few true stem cells. One type of these true stem cells are germline stem cells, or GSCs. These are not always found in females, but are in males and ultimately become the reproductive cell of the organism, be it egg or sperm (Moore 2014). There are GSCs found in males of *Oncopeltus fasciatus*, just as in *Drosophila melanogaster*. In male *D. melanogaster*, the representative insect for germline studies, the system in which these stem cells thrive has been determined. Germline stem cells reside in a defined anatomical niche that is defined by somatic cells, in insects, and the stem cells either self-renew or produce progeny that then become sperm through differentiation. The GSCs that divide to form progeny that undergo multiple rounds of mitotic division then switch to meiotic division to become gametes (Spradling et al. 2011).

In *Oncopeltus fasciatus*, there are two testes with seven testicular follicles which together become covered by a membrane, called the testis envelope. In the apex of the testis follicle there are apical cells that form a sphere, that are then surrounded by the germline stem cells which are then in turn surrounded by the cyst progenitor cells on the outermost part of the tip of the follicle. The apical cells provide the signals that determine if there is maintenance or turnover of the GSCs, both by attachments that the GSCs have to the cells and by molecules

released into the cytoplasmic space. The GSCs have projections that reach out to touch the niche cells because there are a limited number of apical cells, twelve is the usual number, and a continually expanding number of GSCs (Schmidt and Dorn 2004).

In *D. melanogaster*, when GSCs are given the signal to divide, the division is asymmetric meaning that the mitotic spindle is set up perpendicularly to the niche (hub)-GSC interface so that one daughter remains next to the niche and retains the qualities of a stem cell and the other daughter moves away and initiates differentiation (Spradling et al. 2011). However, in *O. fasciatus*, GSCs in first and second instar nymphs undergo mitosis producing two GSCs, which would indicate symmetric division, but then in third instars through adults the mitotic spindle is placed perpendicularly to the to the surface of the apical cells, so there is then asymmetrical division as gonialblasts are being formed (Schmidt, Sehn and Dorn 2001).

Once the gonialblast is separated from its sister cell, it is surrounded by a cyst stem cell (CySC). These cells stay together for the rest of their journey through differentiation. This GSC-CySC pair then undergoes transit amplification mitotic division until there are 16 cells in the spermatocyst. The cells have not undergone complete division are connected by intercellular bridges. This 16-cell spermatocyst then undergoes two meiotic divisions with incomplete cytokinesis, and then 64 spermatids are formed. Sperm tails are elongated and mature sperm is eventually formed (Demarco et al. 2014). Mature sperm is kept in the seminal vesicle until copulation and when transferred to the female, is stored in the spermatheca.

How are GSCs maintained or signaled to divide?

Most studies on maintenance and differentiation of GSCs have been completed on *D. melanogaster* (reviewed in Matunis et al. 2012) and therefore the studies are used as a proxy

for *Oncopeltus fasciatus*. For maintenance of GSCs, apical cells secrete Unpaired, which then activates JAK-STAT signaling in GSCs and CySCs. Another signaling pathway that can maintain the GSCs is BMP, mainly two ligands, *decapentaplegic (dpp)* and *glass bottom boat (gbb)*. These two ligands, *gbb* and *dpp*, are expressed in the niche and CySCs and can cause self-renewal of GSCs. Insulin signaling, from both the brain and testes, can also affect the amount of GSCs and CySCs. If there is not an insulin receptor in GSCs, these cells are not maintained, and constitutive insulin signaling can suppress starvation-mediated loss of GSCs. There are also implications that GSCs can be regulated by epigenetic factors in *D. melanogaster. NURF*, nucleosome remodeling factor complex, *Nclb*, *No child left behind*, and *Phf7*, *PHD Finger Protein 7*, are all required for male maintenance and differentiation in some way.

Zfh-1, *chinmo*, and *ken and barbie* are expressed in the CySCs and are required for their maintenance. In the niche, the ligand hedgehog is required for CySC maintenance (Matunis, Stine and de Cuevas 2012). In *D. melanogaster*, the niche cells are maintained by *FOXO*, *Forkhead Box protein O*, by way of *fringe*, to control *notch* signaling depending on the level on insulin (Yang et al. 2013). As these pathways have not been studied in *O. fasciatus,* we can use the conserved germline stem cell pathways from other models studied (Terry et al. 2006, Matunis et al. 2012, Yang et al. 2013, Zhang et al. 2013), and predict that homologues of these genes in *O. fasciatus* will be involved in GSC maintenance.

DIET AND LIFE HISTORY

Life History

The life history of an individual is the life, from birth to death, in which patterns of reproduction, survival, maturation, and death are described. Reproductive success in organisms

is what we want to understand, given either ecological selection or internal trade-offs and constraints. The traits in life history are related to the environment, and therefore developmental plasticity, where one genotype creates multiple phenotypes, is a main player in life history studies (Flatt and Heyland 2011). This organism is easy to use in these types of studies and has been used in these studies for the past few years, because it has a short generation time and the ability to manipulate their life history in the lab.

Diet and its role

The effect of diet on life history traits, be it reproduction or longevity, has been studied for many years (e.g. Mair et al. 2010). These studies have looked at dietary restriction, in the form of protein restriction (Mair et al. 2010; McLeod et al. 2010; Davila and Aron 2017), and how it affects the lifespan of individuals on a protein limited diet. Mair et al. (2010) found that restricting the diet of male *Drosophila melanogaster* not only extended the lifespan of the flies, but also increased the number of germline stem cells found in the testis. In McLeod et al. (2010), feeding males a diet lacking in protein caused a reduction in spermatogenesis, this was due to a decrease in GSC proliferation which was rescued when the males were refed. It was also determined that the niche cells could respond to insulin signaling and coordinate changes in stem cells and their behavior depending on metabolic flux. In ants, restricting the protein in the diet during development affected the amount of sperm that was produced, but viability was not affected (Dávila & Aron 2017). Lipids, another major component of any diet, and their metabolism has been implicated in the regulation of lifespan extension related to diet. In *C. elegans*, nuclear hormones control cholesterol homeostasis or the insulin/insulin-like growth

factor signaling (IIS) pathway which can in turn extend the lifespan of an individual (Bustos and Partridge 2017).

Germline stem cells, as some of the only stems cells an adult has, can be an indicator for the health of an organism. As males age, or if eating a less than nutritious meal, there are fewer GSCs that can be found in the follicle (McLeod et al. 2010). As our organisms can eat different seeds that are different in their nutritional content (Nation and Bowers 1982), we can use the variation to identify genes and mechanisms that are involved in spermatogenesis and GSC maintenance and differentiation in *Oncopeltus fasciatus*. The laboratory *O. fasciatus* eat sunflower seeds and have been eating these seeds for generations, but they can always eat the ancestral diet, or diet in the wild, of milkweed seeds.

These diets are different in that the amount of polyunsaturated fatty acids (PUFAs) and other fatty acids that can be found in the bodies of *Oncopeltus* when having eaten these seeds may be altered. Males that have eaten sunflower seeds have a higher amount of oleic acid (C18:1 cis-9) and males having eaten milkweed seeds have higher amounts of steric acid (C18) (Nation and Bowers 1982). PUFAs do impact the insulin-signaling pathway and, it could then impact the GSCs. It has also been shown that PUFAs affect longevity and reproduction in *Caenorhabditis elegans* and *D. melanogaster* (Vrablik and Watts 2013). These seeds also have a different percent protein content and that could also play a role in life history of these organisms. Males of *O. fasciatus* have been fed these two diets (sunflower- and milkweed-seeds) in the lab before and it was shown that males fed milkweed seeds invested more into mating and fertility than longevity, while their sunflower-fed counter parts invested in longevity at the expense of mating and fertility (Attisano et al. 2012).

Life History and Transcriptomics

The Etges lab (e.g. Etges 2014) has used transcriptomes to describe differences in life history traits at different life stages and on different diets multiple times, and that is the way we plan in using our transcriptome. In Crowley-Gall et al. (2016), they show that different environments can affect the transcription profiles of *Drosophila mojavensis*. This is one example of phenotypic plasticity that can be recognized by a transcriptomic study. We want to try and determine if this phenotypic plasticity seen in *Oncopeltus fasciatus* on different diets can been seen in the transcriptome, like in *D. mojavensis*, and if a difference in the genes can be the ultimate cause in the phenotypic change.

HOW DOES IT ALL TIE TOGETHER?

One central question tries to bring everything together. How does the diet of an organism, specifically *Oncopeltus fasciatus*, effect the life history of reproduction and longevity and the underlying mechanisms? We chose to approach this question from two different angles: phenotypically and genotypically; so, by the outward showing of traits, including fertility, fecundity, sperm and spermatocyst counts, and by completing a transcriptome of the testis. Both having diet as the ultimate comparison between groups.

Underlying this main question, there are smaller questions within each study. The phenotypic study included what one would normally see in a life history study. A change in diet looking at traits fertility, fecundity, and counts of sperm and spermatocysts. This study tells us if diets can change these life history traits, if phenotypic plasticity does occur due to diet. Some of the questions we wanted to ask included: How does diet affect the fertility and fecundity of males fed different diets; How does the difference in diet affect the sperm, quality or quantity;

and can we determine if this difference can be seen in the germline stem cells or the spermatocysts?

The genotypic study was a transcriptome that tried to elucidate the different transcripts between the diets for either GSC turnover/maintenance or spermatogenesis. We looked at three main questions, as this was mainly a question generating project. First, what are transcriptional differences associated with the different biological activities that take place in the different regions of the testes, or the tip of the testis and the distal testis. Second, we compared the transcriptional profiles of the testis tips of milkweed and sunflower-fed males. Finally, we compared the transcriptional profiles of the distal region of the testis between the two diets.

REFERENCES

Burdfield-Steel, Emily R., and David M. Shuker. 2014. "The Evolutionary Ecology of the Lygaeidae." Ecology and Evolution. doi:10.1002/ece3.1093.

Bustos, Victor, and Linda Partridge. 2017. "Good Ol' Fat: Links between Lipid Signaling and Longevity." Trends in Biochemical Sciences xx. Elsevier Ltd: 1–12. doi:10.1016/j.tibs.2017.07.001.

Crowley-Gall, Amber, Priya Date, Clair Han, Nicole Rhodes, Peter Andolfatto, John E Layne, Stephanie M Rollmann, et al. 2016. "Population Differences in Olfaction Accompany Host Shift in Drosophila Mojavensis." Proceedings. Biological Sciences / The Royal Society 283 (1837): 101–9. doi:10.1098/rspb.2016.1562.

Dávila, Francisco, and Serge Aron. 2017. “Protein Restriction Affects Sperm Number but Not Sperm Viability in Male Ants.” Journal of Insect Physiology 100 (May). Elsevier: 71–76. doi:10.1016/j.jinsphys.2017.05.012.

Demarco, R. S., A. H. Eikenes, K. Haglund, and D. L. Jones. 2014. “Investigating Spermatogenesis in Drosophila Melanogaster.” Methods 68: 218–27. doi:10.1016/j.ymeth.2014.04.020.

Etges, William J. 2014. “No Boundaries: Genomes, Organisms, and Ecological Interactions Responsible for Divergence and Reproductive Isolation.” In Journal of Heredity, 105:756–70. doi:10.1093/jhered/esu039.

Feir, Dorothy. 1974. “Oncopeltus Fasciatus: A Research Animal.” Annual Review of Entomology, no. 144: 81–96. doi:10.1146/annurev.en.19.010174.000501.

Flatt, Thomas, & Andreas Heyland. 2011. Mechanisms of Life History Evolution. Oxford University Press. doi:10.1093/acprof:oso/9780199568765.001.0001.

Grandison, Richard C., Matthew D. W. Piper, and Linda Partridge. 2009. “Amino-Acid Imbalance Explains Extension of Lifespan by Dietary Restriction in Drosophila.” Nature 462 (7276): 1061–64. doi:10.1038/nature08619.

Liu, Paul, and Thomas C. Kaufman. 2009. “Morphology and Husbandry of the Large Milkweed Bug, Oncopeltus Fasciatus.” *Cold Spring Harbor Protocols*. doi:doi:10.1101/pdb.emo127.

Mair, William, Catherine J. McLeod, Lei Wang, and D. Leanne Jones. 2010. “Dietary Restriction Enhances Germline Stem Cell Maintenance.” Aging Cell 9 (5): 916–18. doi:10.1111/j.1474-9726.2010.00602.x.

Matunis, Erika L, Rachel R Stine, and Margaret de Cuevas. 2012. “Recent Advances in Drosophila Male Germline Stem Cell Biology.” Spermatogenesis 2 (3): 137–44. doi:10.4161/spmg.21763.

McLeod, Catherine J., Lei Wang, Chihunt Wong, and D. Leanne Jones. 2010. “Stem Cell Dynamics in Response to Nutrient Availability.” Current Biology 20 (23). Elsevier Ltd: 2100–2105. doi:10.1016/j.cub.2010.10.038.

Misof, B., S. Liu, K. Meusemann, R. S. Peters, A. Donath, C. Mayer, P. B. Frandsen, et al. 2014. “Phylogenomics Resolves the Timing and Pattern of Insect Evolution.” *Science* 346 (6210): 763–67. doi:10.1126/science.1257570.

Moore, PJ. 2014. “Reproductive Physiology and Behaviour.” *The Evolution of Insect Mating Systems*, 1–24.

Nation, J. L, and W. S. Bowers. 1982. “Fatty Acid Composition of Milkweed Bugs Oncopeltus Fasciatus and the Influence of Diet.” Insect Biochemistry 12 (4): 455–59. doi:0020-1970/82/04455-05$03.00/0.

Schmidt, Esther D., and August Dorn. 2004. “Structural Polarity and Dynamics of Male Germline Stem Cells in the Milkweed Bug (Oncopeltus Fasciatus).” Cell and Tissue Research 318 (2): 383–94. doi:10.1007/s00441-004-0983-6.

Schmidt, Esther D., Elisabeth Sehn, and August Dorn. 2002. “Differentiation and Ultrastructure of the Spermatogonial Cyst Cells in the Milkweed Bug, Oncopeltus Fasciatus.” Invertebrate Reproduction and Development 42 (2–3): 163–78. doi:10.1080/07924259.2002.9652773.

Spradling, A., M. T. Fuller, R. E. Braun, and S. Yoshida. 2011. “Germline Stem Cells.” Cold Spring Harbor Perspectives in Biology 3 (11): a002642. doi:10.1101/cshperspect.a002642.

Terry, Natalie A, Natalia Tulina, Erika Matunis, and Stephen DiNardo. 2006. “Novel Regulators Revealed by Profiling Drosophila Testis Stem Cells within Their Niche.” *Developmental Biology* 294 (1): 246–57. doi:10.1016/j.ydbio.2006.02.048.

Vrablik, Tracy L., and Jennifer L. Watts. 2013. “Polyunsaturated Fatty Acid Derived Signaling in Reproduction and Development: Insights from Caenorhabditis Elegans and Drosophila Melanogaster.” Molecular Reproduction and Development 80 (4): 244–59. doi:10.1002/mrd.22167.

Yang, Sheng An, Wen Der Wang, Ciao Ting Chen, Chen Yuan Tseng, Yi Ning Chen, and Hwei Jan Hsu. 2013. “FOXO/Fringe Is Necessary for Maintenance of the Germline Stem Cell Niche in Response to Insulin Insufficiency.” Developmental Biology 382 (1): 124–35. doi:10.1016/j.ydbio.2013.07.018.

Zhang, Zhao, Xiangdong Lv, Jin Jiang, Lei Zhang, and Yun Zhao. 2013. “Dual Roles of Hh Signaling in the Regulation of Somatic Stem Cell Self-Renewal and Germline Stem Cell Maintenance in Drosophila Testis.” *Cell Research* 23: 1–4. doi:10.1038/cr.2013.29.

CHAPTER 2

A STUDY OF THE TRANSIT AMPLIFICATION DIVISIONS DURING SPERMATOGENESIS IN *ONCOPELTUS FASCIATUS* TO ASSESS PLASTICITY IN SPERM NUMBERS OR SPERM VIABILITY UNDER DIFFERENT DIETS[1]

[1] Duxbury, A.E., B. Weathersby, Z. Sanchez, and P.J. Moore. Submitted to *Ecology and Evolution*.

ABSTRACT

Oncopeltus fasciatus males fed the ancestral diet of milkweed seeds prioritize reproduction over lifespan as evidenced by higher rates of fertility and shorter lifespans than males from the same population fed the adapted diet of sunflower seeds. We examined the proximate mechanisms by which milkweed-fed males maintained late-life fertility. We tested the hypothesis that older milkweed-fed males maintained fertility by producing more, higher quality sperm. Our results, that older males have more sperm, but their sperm do not have higher viability is in general agreement with other recent studies on how nutrition affects male fertility in insects. We further examined the mechanisms by which sperm are produced by examining the progression of spermatogonial cells through the cell cycle during transit amplification divisions. We demonstrated that diet affects the likelihood of a spermatocyst being in the S-phase or M-phase of the cell cycle. Given work in model systems, these results have implications for subtle effects on sperm quality either through replication stress or epigenetic markers. Thus, viability may not be the best marker for sperm quality and more work is called for on the mechanisms by which the germline and the production of sperm mediate the cost of reproduction.

INTRODUCTION

A fundamental tenant of life history theory is that there is a trade-off between reproduction and lifespan (Stearns 1992). However, the mechanisms by which the cost of reproduction is manifest have been elusive (Speakman 2008, Harshman & Zera 2007, Flatt 2011). Few studies have identified the mechanisms underlying phenotypic plasticity in the trade-off under different environmental conditions, even though we expect individuals to have the ability to strategically adjust expenditure on gametes given the likelihood that a particular reproductive opportunity will result in fitness benefits. The best-studied example is dietary restriction, defined as reduced food without malnutrition. Dietary restriction leads to an increase in lifespan, usually with a reduction in reproductive rates, and we assume that this plasticity is adaptive, allowing organisms to maximize fitness to specific environmental conditions (e.g. Zajitschek et al. 2016).

Much of the work on the physiology of the reproduction-lifespan trade-off has focused on females, and the cost of producing expensive gametes (Hayward & Gillooly 2011), yet males also experience significant costs of reproduction. Research on reproductive costs for males has focused on mate searching, courtship, and male-male competition under different environments, both social and nutritional (Hunt et al. 2004, Flatt & Heyland 2011, Scharf et al. 2013, Shuker & Simmons 2014). It is now clear, however, that the sperm production represents a significant cost to males and researchers are exploring phenotypic plasticity in sperm numbers and quality under variable social and nutritional environments (Moatt et al. 2014, Bunning et al. 2015, Joseph et al. 2016, Dávila & Aron 2017). *Drosophila melanogaster* males exposed to the odor of a rival male store both more sperm and a greater proportion of live

sperm in their seminal vesicles (Moatt et al. 2014). High quality nutrition, on the other hand, appears to promote increased sperm numbers, but does not impact sperm quality in cockroaches (Bunning et al. 2014), leaf-footed bugs (Joseph et al. 2016), or ants (Dávila & Aron 2017).

While all of these studies document an outcome of environmental variation on sperm quantity and quality, none examined the mechanisms by which the increase in sperm numbers occurred. Ultimately sperm availability depends on the germline, cells set aside for the production of gametes (Extavour 2013, Moore 2014). Males have the potential, through germline stem cells, to modulate sperm production (Kaczmarczyk & Kopp 2011, Moore 2014). While we have many studies examining the developmental and genetic controls on germline stem cells, these cells are rarely examined in an evolutionary context. Further, the energetic cost of producing gametes may not represent the full, or even major, cost of reproduction (Maklalov & Immler 2016). Maintaining genomic and proteomic integrity within the germline may be more costly in males than females, given the increased rate of turnover in the germline stem cells required for producing numerous sperm.

Laboratory populations of the milkweed bug, Oncopeltus fasciatus, are able to utilize both the ancestral food of milkweed seeds but also an adapted diet of sunflower seeds (Moore & Attisano 2011, Newcombe et al. 2015a, 2015b). Females show no difference in fitness on the two diets (Moore & Attisano 2011). Males, however, while having equal lifetime reproductive success, demonstrate different patterns of life history trade-offs on the two diets (Attisano et al. 2012). Milkweed-fed males prioritize reproduction over lifespan, investing both in increased

mating behavior but also by maintaining late life fertility. Older milkweed-fed males fertilize a greater proportion of their mate's eggs than older sunflower-fed males.

In this study, we tested the hypothesis that reduced late-life fertility in sunflower-fed males was due to reduced sperm production, reduced sperm quality, or a combination of reduced sperm numbers and sperm quality. We also examined the developmental mechanism which could give rise to any potential change in sperm numbers. Spermatogenesis requires a series of events, any one of which could be affected by diet (Figure 2.1). Variation in the rate of germline stem cell division to produce spermatogonial cells, the rate at which the spermatogonial cells undergo transit amplification divisions to form spermatocysts, or the rate of entry into meiosis to produce spermatocytes will result in variation in the rate of sperm production. We predicted that older milkweed-fed males would have a higher sperm viability than sunflower-fed males. We also predicted that older milkweed-fed males would have more sperm stored in their seminal vesicles due to an increase in the rate of transit amplification divisions.

METHODS

Animal Husbandry

All colonies and individuals were kept at 26°C and 16:8 L:D. Eggs were collected from mass colonies and left to mature through 5^{th} instar in a nymphal colony with sunflower seeds and water. On the day of adult emergence, experimental males were put into individual dishes with either organic, unsalted sunflower seeds (FoodtoLive.com; sunflower-fed) or milkweed seeds (Everwilde.com; milkweed-fed) and water. Newly emerged females were put into

colonies with sunflower seeds and water and kept to provide males with virgin females as mates.

Experimental males were all mated at 2 weeks, 3 weeks and at 4 weeks post-adult eclosion. Mating trials were carried out as described in Attisano et al. 2011. The food was removed from the male's dish to prevent any effect of food treatment on female fecundity. A female was then introduced to the dish. All pairs were observed until the first mating. Pairs were placed back into the incubators and were allowed to mate over a period of 48 hours.

Fertility and fecundity

For the 2- and 4-week mating trials in which male fertility was being assessed, we controlled for effect of female age by using females that were 7-10 days post-adult eclosion and were virgins at the time of the mating trials. The females for the 3-week mating trials were of unknown age and mating status, but all mated within the first few hours of the mating trial. These females were returned to the mass colony following the mating trial. The virgin females from the 2-weed and 4-week mating trials were placed in individual petri dishes with water, sunflower seeds, and cotton wool for laying eggs after their 48-hour mating with the experimental male. Oncopeltus fasciatus eggs are pale yellow when they are laid and develop a deep red color as the embryo develops, allowing embryo development to be scored visually. Eggs from the female were collected every 3 days and left in the incubator to mature until they reached a red color, usually 5 days, indicating they were fertilized and had initiated development. Eggs were then counted and scored as fertilized or not fertilized based on their color. The females were left to lay eggs for the rest of their lives, which lasted about 4 to 5

weeks, and egg numbers combined to examine lifetime fecundity (total eggs produced) and fertility (percent of eggs fertilized) for mates of sunflower- and milkweed-fed males.

Quantity and quality of sperm

To determine if diet affects either the quantity or quality of sperm produced by males, we assessed both the numbers of sperm stored in the seminal vesicle and viability of stored sperm of 4-week post eclosion males on each diet. Males in this experiment had only mated once and then given 10 days to recover sperm stores prior to dissection. Males were dissected into PBS and one seminal vesicle was used for sperm quantity and the other for sperm quality assays.

Sperm quantity was determined by counting the number of sperm from the seminal vesicle (Montrose et al. 2008). The seminal vesicle was placed into 200 ultrapure water and gently ground with a micro-pestle. A 4 µL aliquot of the sperm was diluted into 600 µL ultrapure water and 10 µL of 0.5% EosinY was added to the dilution to improve contrast on the slide. The diluted sperm was then placed onto slides in a series of ten 10 µL spots. The spots were allowed to dry and all sperm in the 10 spots were counted. The total number of sperm from the seminal vesicle was calculated.

Sperm quality was assayed using the LIVE/DEAD Sperm Viability kit (Montrose et al. 2008; ThermoFisher Scientific). This kit utilizes a cell membrane permeable green fluorescent stain (SYBR 14) and the membrane impermeable stain propidium iodide to differentiate between living and dead cells. Living cells are labeled green due to the presence of the SYBR 14 and the exclusion of the propidium iodide (see Figure 2A). Dead or dying cells are unable to exclude the propidium iodide and so are stained red. The second seminal vesicle was placed in

1 mL TIB + 10% bovine serum albumin and gently broken open with a micropestle. 100 µL of the sperm solution was placed into 900 µL of testis incubation buffer (TIB; 183 mM KCl, 47mM NaCl, 10mM Tris, pH 6.9: Parrott et al. 2012) and SYBR 14 and PI added to the sperm solution. After 10 minutes at RT, 12 samples from each seminal vesicle were placed on a welled slide and imaged with an AMG EVOS FL microscope. Slides were imaged with both the GFP (showing the SYBR 14 staining) and RFP (showing the propidium iodide staining) filter sets and one image containing at least 10 sperm cells were taken of each of the 12 wells. Images were randomized and counted blind. The number of living (green) and dead/dying (red) cell were counted for each male.

Germline staining

Males were chosen randomly to be dissected at young age (one day after the 2-week mating trial), or old age (one day after the 4-week mating trial). Testes were removed from the male and placed into TIB. Individual testioles were separated and removed from the surrounding testis membrane prior to fixation. Testioles were assayed ex vivo for cell proliferation using two different markers of the cell cycle. First, we assayed for cells in the S-phase using the Click-iT EdU Alexa Fluor 647 imaging kit (Thermo Fisher Scientific C10340). EdU is incorporated into DNA during the S-phase of the cell cycle and is then visualized with a fluorescent tag. We also stained testioles for cells in the M-phase of the cell cycle using a polyclonal antibody against Histone H3 phosphorylated at serine 10 (pHH3), a modification specific to the mitotic phase of the cell cycle (Millipore Sigma Antibody 06-570). Labeling two phases of the cell cycle allows researchers to distinguish between variation in length of the entire cell cycle (in which the change observed should be consistent among the two markers) or

variation in a single phase of the cell cycle (in which the results with two markers will be discordant).

Individual testioles were incubated in TIB and EdU for 45 minutes at RT to allow for incorporation of EdU into cells in the S phase of the cell cycle. After EdU incorporation, testioles were fixed in 4.5% formaldehyde in phosphate saline buffer (PBS) for 30 minutes at RT. Following washes in PBS plus 0.1% Tween-20 (PBT) and 5% normal goat serum, the EdU was labeled with the 647 (cyan) fluorescent ClickiT reagent for 30 minutes in the dark at RT. Testioles were then incubated with the primary pHH3 antibody, followed by a goat anti-rabbit secondary antibody labeled with an Alexa Fluor 488 fluorescent (green) marker.

The stained testes were then imaged using a Zeiss LSM 710 Confocal Microscope (Zeiss) at the UGA Biomedical Microscopy Core and an EVOS Fl Cell Imaging system (Thermo Fisher). Three testioles from each male were imaged and analyzed. Images were coded and counted blind by two independent operators. For each testiole the number of spermatocysts stained positive for anti-pHH3 antibody or EdU was counted and the total for all three testioles from each male added together (see Figure 2B).

Statistical Analyses

Statistical analyses were done using JMP Pro v 13.0.0. For the fertility and fecundity experiment, male reproductive success was assessed across two ages. Therefore, for this experiment we utilized a Repeated Measures ANOVA. The experiment on sperm numbers and sperm viability, only a sperm from the older males was examined. This data was analyzed with a one-way ANOVA using diet as the factor. Analysis of the cell cycle was carried out on both 2-week and 4-week old males, but due to the destructive sampling required each age represents

a different group of males. Therefor the data was analyzed with a two-way ANOVA using diet and age as factors.

RESULTS

Fertility and fecundity of females based on male diet

In this experiment, the fecundity of females mated to young (2-week-old) males was statistically significantly higher than those mated to old (4-week-old) males (within-subjects, Table 2.1; Figure 2.3A). However, there was no statistically significant difference in fecundity of females due to the diet of their mates and no interaction between age and diet (between-subjects, Table 2.1). Age did not statistically significantly affect the fertility of males on either diet (within-subjects, Table 2.1; Figure 2.3B), but diet did have a statistically significant effect on fertility (between-subjects, Table 2.1). Milkweed-fed males had higher fertility than sunflower-fed males at both ages and there was no interaction between age and diet (Table 2.1).

Quality and quantity of sperm

Old milkweed-fed males had statistically significantly greater numbers of sperm stored in their seminal vesicles than old sunflower-fed males ($F_{1, 38} = 5.225$, $p = 0.028$; Figure 2.4A). Diet did not affect the viability of the sperm within the seminal vesicle however ($F_{1, 12} = 1.400$, $p = 0.260$; Figure 2.4B). The viability of sperm isolated from the seminal vesicle from both milkweed-fed and sunflower-fed males was close to 100%.

Germline division rates

The effect of diet and age on the phase of the cell cycle of spermatogonial cells within the testis tubules was complex. There was no statistically significant effect of either diet or age, and no statistically significant interaction between diet and age, for the total number of

spermatocysts that that are dividing and thus stain positive for either S-phase or M-phase (EdU and anti-pHH3 stained spermatocysts combined; Table 2.2). There was a statistically significant effect of diet on total numbers of spermatocysts staining positive with the mitosis stage specific marker, anti-pHH3 antibody (Table 2.2). Overall, sunflower-fed males have more spermatocysts that stain positive for mitosis than milkweed-fed males (Figure 2.5A). There was no statistically significant effect of age and no statistically significant interaction between diet and age (Table 2.2). For the marker of the DNA synthesis (S-) phase of the cell cycle, EdU incorporation, there was a statistically significant effect of diet (Table 2.2; Figure 2.5B). There was no statistically significant effect of age (Table 2.2), but there was a statistically significant interaction between age and diet. Interestingly, milkweed-fed males are much more likely to have spermatocysts that stain positive for S-phase within young males, although that difference goes away with age (Figure 2.5B).

If the rate of sperm production under the two diets varied due to a simple speeding up or slowing down of the cell cycle proportionally, the results using the markers for S-phase and M-phase should be the same for the treatments, which was not the case. Thus, the relationship between the two different stages of the cell cycle was investigated further by testing for a correlation between the two different stains. In the 2-week males, there is a statistically significant negative correlation between the total number of spermatocysts staining positive for Edu and anti-pHH3 ($r = -0.601$, $p = 0.008$), indicating that spermatocysts at this age are dividing in relative synchrony and are more likely to be observed in the M-phase than the S-phase of the cell cycle. This correlation goes away in the 4-week males ($r = 0.099$, $p = 0.542$).

DISCUSSION

In laboratory populations of the milkweed bug Oncopeltus fasciatus males fed the ancestral diet of milkweed seed prioritize reproduction over lifespan (Attisano et al. 2012). Here we examined the mechanism responsible for maintaining fertility later in life in these males. We asked whether older milkweed-fed males maintain sperm production as compared to males fed the adapted diet of sunflower seeds. Additionally, given the increasing evidence that sperm quality declines with age, we asked whether or not older milkweed-fed males maintain their fertility through increased sperm quality. Our results support the hypothesis that older milkweed-fed males maintain late-life fertility by maintaining sperm numbers. However, older milkweed-fed males did not have higher sperm quality, measured as viability, than sunflower-fed males. These results are in general agreement with other recent studies on how nutritional variation impacts male fertility in insects (Bunning et al. 2015, Joseph et al. 2016, Dávila & Aron 2017), where diet influences sperm numbers or testis mass, but not sperm viability.

Although our experimental design was slightly different, our results generally agreed with Attisano et al. (2012). As in Attisano et al. (2012), females mated to older males had lower fecundity. Milkweed-fed males had higher fertility than sunflower-fed males. The difference in fertility documented in Attisano et al. (2012) and here can be explained by an increase in the numbers of sperm stored in the seminal vesicle of older milkweed-fed males. While there are a growing number of studies documenting the effect of nutrition on sperm quantity and quality in insects (see citations in Bunning et al. 2015), fewer have examined the consequences of this variation on male fitness. In this study, we observed a correlation among the two diets

between higher male fitness (measured as percent of eggs fertilized) and the increase in sperm production. Thus, as was recently observed in the cockroach *Nauphoeta cinere*a (Bunning et al. 2015) and the flour beetle *Tribolium castaneum* (Fedina & Lewis 2006), sperm production appears to directly translate into fitness. However, that is not the case in all systems. An increase in sperm production on a high-quality diet in *D. melanogaster* did not result in higher paternity (McGraw et al. 2007), although this study was the only one of these to examine larval as opposed to adult nutritional environment. Adult diet may be particularly important in insects where spermatogenesis proceeds throughout adulthood (Joseph et al. 2016).

Male fertility is affected by sperm quality as well as numbers. It is becoming increasingly clear that sperm quality can decline with age and environmental conditions males experience (Marshall 2015, Pizzari et al. 2008). One way milkweed-fed males may maintain late-life fertility is through maintaining sperm quality. Therefore, we tested for changes in sperm viability among the older males fed the two diets. Sperm viability was uniformly high across the diets. This corresponds to what we might predict for species that are likely to experience high levels of sperm competition (Hunter & Birkhead 2002), such as *O. fasciatus*, which mates promiscuously. Thus, the higher fertility in males fed the ancestral diet of milkweed seeds over those fed the adapted diet of sunflower seeds appears at the surface to be mediated through maintaining sperm production rather than maintaining sperm quality. However, as we discuss below, sperm viability may not be the best way of measuring sperm quality.

Clearly sperm production is costly, and as such diet will have an impact on sperm production and fertility (e.g. Bunning et al. 2015). Sperm production is influenced by energy acquisition. Bunning et al. (2015) show that in the cockroach *N. cinerea* sperm production, but

not sperm viability, increased with increased intake of nutrients. The same response was documented in male ants (*Linepithema humile*), where decreasing protein intake results in decreasing sperm numbers without a change in sperm viability (Dávila & Aron 2017). But how does this affect the trade-off between reproduction and lifespan? Variation in reproductive effort, such as variation in sperm production, do not inevitably result in a change in lifespan, and yet this negative correlation between fertility and lifespan is ubiquitous. Survival costs of reproduction could arise through competitive allocation of a limited resource pool, direct costs via damage to the soma such as accumulation of reactive oxygen species, or antagonistic signaling between the germline and the soma (Kaczmarczyk & Kopp 2010, Flatt 2011, Edward & Chapman 2013, Aguilaniu 2015, Maklakov & Immler 2016). Alternatively, nutritional environment could cause a change in the physiological state of the organism that has independent, but opposite, effects on reproduction and lifespan (Aguilaniu 2015).

Milkweed-fed males maintain late life fertility, but pay a cost by reduced lifespan (Attisano et al. 2012). Milkweed-fed males aren't simply better fed, and thus able to invest in more sperm. Rather, the ancestral diet of milkweed is altering the life-history trade-off between reproduction and lifespan. It has been argued that to better understand the nature of this trade-off we need to understand the proximate mechanisms underlying it (Harshman & Zera 2007, Flatt 2011, Hansen et al. 2013, Aquilaniu 2015). We examined the developmental mechanisms of sperm production under the two diets. Our results were not clear cut. While the number of sperm stored within the seminal vesicles was significantly different, there was no difference in the numbers of spermatocysts undergoing transit amplification divisions by either age or diet. Diet did, however, influence the stage of the cell cycle that we were likely to

detect in the testioles of males. Thus, the spermatocysts in the testes of milkweed-fed males were more likely to be in the S-phase of the cell cycle than sunflower-fed males, while spermatocysts of sunflower-fed males were more likely to be in the M-phase. And while we did not find an overall effect of age on transit amplification divisions, we found that younger males tended to have spermatocysts dividing synchronously, while that synchrony breaks down in the older males.

Our result on transit amplification divisions within the testioles are not easily reconciled with our phenotypic results on sperm numbers. We think there may be several reasons that could account for this. First, because these males were not mating frequently, the sperm stored in the seminal vesicles could have come from spermatogenesis occurring across the males' lifetime. Thus, our sperm counts on older males would reflect spermatogenesis that occurred at younger ages. Second, while the rate of transit amplification divisions is one avenue to produce variation in sperm numbers, other steps in the process may also impact sperm production (Figure 2.1). While the germline stem cells and stem cell niche have been identified morphologically in *O. fasciatus* (Schmidt et al. 2002, Schmidt & Dorn 2004), we do not currently have the molecular markers to identify the male germline stem cells or the stem cell niche, and thus we are unable to measure variation in the rate at which spermatogonial cells are born (Figure 2.1, step 1) as has been done in *Drosophila melanogaster*. In *D. melanogaster*, the numbers of germline stem cells in the testes vary with both age (Wang & Jones 2010) and diet (McLeod et al. 2010, Wang et al. 2011). Thus, stem cells can respond directly to the nutritional status and thus represent a potential avenue for coordinating diet and fertility (Kaczmarczyk & Kopp 2011, Moore 2014). We are working to identify cell markers such that we

can directly assess germline stem cell dynamics rather than using the indirect measure of transit amplification division rates. Variation in the rate at which spermatocysts transition to meiosis also could affect sperm numbers (Figure 2.1, step 3). Deleted in Azoospermia is a highly-conserved gene family involved in male fertility (VanGompel & Xu 2011). The ancestral gene in the family, *boule*, is found in invertebrates, including *O. fasciatus* (Ewen-Campen et al. 2013) and a threshold level of Boule protein is required for the progression of spermatogonia into meiotic divisions (VanGompel & Xu 2011). We have preliminary evidence that boule expression is upregulated in the testes of sunflower-fed males (Duxbury et al. unpublished data). If the threshold of Boule protein is reached in the spermatogonia of sunflower-fed males earlier, it would result in spermatogonia dividing meiotically to form spermatocytes after fewer transit amplification divisions and thus result in fewer sperm cells. Further work on how testes dynamics under variable nutrient environments is needed to determine exactly how diet impacts sperm numbers in these males.

While it was unclear how milkweed-fed males produce higher sperm numbers from our data on the cell cycle, the observation that milkweed-fed males have spermatogonia that spend more time in the S-phase of the cell cycle is of interest in terms of sperm quality. It has recently been proposed that the cost of the cellular mechanisms for quality control and repair required to maintain the germline integrity may represent a hidden cost of reproduction (Maklakov & Immler 2016). The variation in progression through transit amplification divisions may result because one diet, sunflower seeds, induces a physiological state that prioritizes somatic maintenance over germline integrity and could reduce fitness not by reducing gamete production but by reducing gamete quality (but not viability) and thus offspring viability. The

mechanism by which environment and age may affect sperm quality is unclear, but emerging evidence in humans indicates that tissue specific changes in epigenetics may influence sperm quality. Genome-wide analysis has documented hyper-methylation of DNA in poor quality sperm and the epigenome is affected by both age and nutrition (Sharma et al. 2015). In vitro fertilization is more likely to result in a successful pregnancy if methylation in sperm is low, although there is no change in fertilization rates. If milkweed-fed males maintained late-life fertility simply by maintaining the cell cycle and the rate of transit amplification divisions, we would expect both of the cell cycle markers to be increased in milkweed-fed males compared to sunflower-fed males. The observation that there are more spermatocysts that stain positive for the S-phase of the cell cycle in milkweed-fed males while there are fewer that stain positive for the M-phase indicates that there is a change in the progression through the cell cycle. One potential explanation for fewer spermatogonia in the S-phase in sunflower-fed males is that the transit amplification divisions in spermatogonia are delayed at the S-phase checkpoint in these males. Replication stress, which can be caused by nutritional limitations, will activate the S-phase checkpoint (Mirkin & Mirkin 2007). As the replication fork stalls, the unwound DNA is vulnerable to damage. Another possibility is that the spermatogonia of milkweed-fed males spend more time synthesizing their DNA and perhaps this improves the efficacy or fidelity of the replication of epigenetic marks (Kheir & Lund 2010). Both of these mechanisms could result in the sperm of milkweed-fed males being of higher quality, measured as the ability to support offspring development as opposed to sperm viability.

CONCLUSION

Oncopeltus fasciatus males show phenotypic plasticity in the reproduction-lifespan trade-off under variable nutritional environments (Attisano et al. 2012). Given that genetic and developmental tools exist for *O. fasciatus*, this system represents an opportunity to examine the proximate mechanism underlying this central life history trade-off. Here we have examined the developmental progression of spermatocysts to explore how variation in nutritional environment might result in variation in sperm numbers, and ultimately fitness of the males. What we have demonstrated is that the pathway from diet to sperm production is not simple. Males do not simply speed up the assembly line. The results from the cell cycle markers, along with increased understanding of how sperm quality can vary with age and environment, lead us to speculate that sperm viability may not be the best measure of sperm quality leading to male fitness. However, O. fasciatus provides an additional model for which we can use a molecular toolkit to untangle proximate mechanisms underlying the cost of reproduction.

ACKNOWLEDGEMENTS

The authors thank Allen J. Moore for advice on statistical analyses. Zachary Sanchez received financial support for his participation in this research from a UGA College of Agriculture and Environmental Sciences Undergraduate Research Award.

AUTHOR CONTRIBUTIONS

The project was conceived and designed jointly by AED and PJM. AED and PJM jointly analyzed the data. AED wrote the original draft of the manuscript with assistance from PJM. BW collected and contributed to the analysis of the data on fecundity and fertility (Figure 2.3) and ZS collected and contributed to the analysis of the data on seminal vesicle sperm counts

and sperm viability (Figure 2.4). Both BW and ZS worked in the laboratory for research credit as a part of their undergraduate degrees.

REFERENCES

Aguilaniu, Hugo. 2015. "The Mysterious Relationship between Reproduction and Longevity." *Worm* 4 (2): e1020276. doi:10.1080/21624054.2015.1020276.

Attisano, A., A. J. Moore, and P. J. Moore. 2012. "Reproduction-Longevity Trade-Offs Reflect Diet, Not Adaptation." *Journal of Evolutionary Biology* 25 (5): 873–80. doi:10.1111/j.1420-9101.2012.02476.x.

Bunning, Harriet, James Rapkin, Laurence Belcher, C Ruth Archer, Kim Jensen, and John Hunt. 2015. "Protein and Carbohydrate Intake Influence Sperm Number and Fertility in Male Cockroaches, but Not Sperm Viability." *Proceedings. Biological Sciences / The Royal Society* 282 (1802): 20142144. doi:10.1098/rspb.2014.2144.

Dávila, Francisco, and Serge Aron. 2017. "Protein Restriction Affects Sperm Number but Not Sperm Viability in Male Ants." *Journal of Insect Physiology* 100 (May). Elsevier: 71–76. doi:10.1016/j.jinsphys.2017.05.012.

Edward, Dominic A., and Tracey Chapman. 2013. "Life History Variation in Male Mate Choice in Drosophila Melanogaster." *Animal Behaviour* 86 (2): 269–75. doi:10.1016/j.anbehav.2013.05.014.

Ewen-Campen, B., T. E. M. Jones, and C. G. Extavour. 2013. “Evidence against a Germ Plasm in the Milkweed Bug Oncopeltus Fasciatus, a Hemimetabolous Insect.” *Biology Open* 2 (6): 556–68. doi:10.1242/bio.20134390.

Ewen-campen, Ben, Tamsin E M Jones, and Cassandra G Extavour. 2013. “Evidence against a Germ Plasm in the Milkweed Bug Oncopeltus Fasciatus , a Hemimetabolous Insect.” *Biology Open* 2: 556–68. doi:10.1242/bio.20134390.

Extavour, C. G. 2003. “Mechanisms of Germ Cell Specification across the Metazoans: Epigenesis and Preformation.” *Development* 130 (24): 5869–84. doi:10.1242/dev.00804.

Fedina, Tatyana Yu, and Sara M. Lewis. 2006. “Proximal Traits and Mechanisms for Biasing Paternity in the Red Flour Beetle Tribolium Castaneum (Coleoptera: Tenebrionidae).” *Behavioral Ecology and Sociobiology* 60 (6): 844–53. doi:10.1007/s00265-006-0228-7.

Flatt, T, and A Heyland. 2011. “Mechanisms of Life History Evolution.”

Flatt, Thomas. 2011. “Survival Costs of Reproduction in Drosophila.” *Experimental Gerontology* 46 (5): 369–75. doi:10.1016/j.exger.2010.10.008.

Hansen, Malene, Thomas Flatt, and Hugo Aguilaniu. 2013. “Reproduction, Fat Metabolism, and Life Span: What Is the Connection?” *Cell Metabolism*. doi:10.1016/j.cmet.2012.12.003.

Harshman, Lawrence G., and Anthony J. Zera. 2007. “The Cost of Reproduction: The Devil in the Details.” *Trends in Ecology and Evolution*. doi:10.1016/j.tree.2006.10.008.

Hayward, April, and James F. Gillooly. 2011. “The Cost of Sex: Quantifying Energetic Investment in Gamete Production by Males and Females.” *PLoS ONE* 6 (1). doi:10.1371/journal.pone.0016557.

Hunt, John, Luc F. Bussière, Michael D. Jennions, and Robert Brooks. 2004. “What Is Genetic Quality?” *Trends in Ecology and Evolution* 19 (6): 329–33. doi:10.1016/j.tree.2004.03.035.

Hunter, F. M., and T. R. Birkhead. 2002. “Sperm Viability and Sperm Competition in Insects.” *Current Biology* 12 (2): 121–23. doi:10.1016/S0960-9822(01)00647-9.

Joseph, Paul N., Daniel A. Sasson, Pablo E. Allen, Ummat Somjee, and Christine W. Miller. 2016. “Adult Nutrition, but Not Inbreeding, Affects Male Primary Sexual Traits in the Leaf-Footed Cactus Bug Narnia Femorata (Hemiptera: Coreidae).” *Ecology and Evolution* 6 (14): 4792–99. doi:10.1002/ece3.2246.

Kaczmarczyk, A. N., and A. Kopp. 2011. “Germline Stem Cell Maintenance as a Proximate Mechanism of Life-History Trade-Offs? Drosophila Selected for Prolonged Fecundity Have a Slower Rate of Germline Stem Cell Loss.” *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology* 33 (1): 5–12. doi:10.1002/bies.201000085.

Kheir, Tony Bou, and Anders H Lund. 2010. “Epigenetic Dynamics across the Cell Cycle.” *Essays In Biochemistry* 48 (1): 107–20. doi:10.1042/bse0480107.

Maklakov, Alexei A., and Simone Immler. 2016. “The Expensive Germline and the Evolution of Ageing.” *Current Biology*. doi:10.1016/j.cub.2016.04.012.

Marshall, D. J. 2015. “Environmentally Induced (Co)variance in Sperm and Offspring Phenotypes as a Source of Epigenetic Effects.” *Journal of Experimental Biology* 218 (1): 107–13. doi:10.1242/jeb.106427.

McGraw, L A, A C Fiumera, M Ramakrishnan, S Madhavarapu, A G Clark, and M F Wolfner. 2007. “Larval Rearing Environment Affects Several Post-Copulatory Traits in Drosophila Melanogaster.” *Biol Lett* 3 (6): 607–10. doi:10.1098/rsbl.2007.0334.

McLeod, Catherine J., Lei Wang, Chihunt Wong, and D. Leanne Jones. 2010. “Stem Cell Dynamics in Response to Nutrient Availability.” *Current Biology* 20 (23). Elsevier Ltd: 2100–2105. doi:10.1016/j.cub.2010.10.038.

Mirkin, E. V., and S. M. Mirkin. 2007. “Replication Fork Stalling at Natural Impediments.” *Microbiology and Molecular Biology Reviews* 71 (1): 13–35. doi:10.1128/MMBR.00030-06.

Moatt, J. P., C. Dytham, and M. D. F. Thom. 2014. “Sperm Production Responds to Perceived Sperm Competition Risk in Male Drosophila Melanogaster.” *Physiology & Behavior* 131: 111–14. doi:10.1016/j.physbeh.2014.04.027.

Moore, P J, and A Attisano. 2011. “Oosorption in Response to Poor Food: Complexity in the Trade-off between Reproduction and Survival.” *Ecology and Evolution* 1 (1). Univ Exeter,

Coll Life & Environm Sci, Ctr Ecol & Conservat, Penryn TR10 9EZ, Cornwall, England: 37–45. doi:10.1002/ece3.4.

Moore, PJ. 2014. “Reproductive Physiology and Behaviour.” *The Evolution of Insect Mating Systems*, 1–24.

Newcombe, Devi, John Hunt, Christopher Mitchell, and Allen J. Moore. 2015. “Maternal Effects and Maternal Selection Arising from Variation in Allocation of Free Amino Acid to Eggs.” *Ecology and Evolution* 5 (12): 2397–2410. doi:10.1002/ece3.1524.

Newcombe, Devi, Patricia J. Moore, and Allen J. Moore. 2015. “The Role of Maternal Effects in Adaptation to Different Diets.” *Biological Journal of the Linnaean Society* 114 (1): 202–11. doi:10.1111/bij.12408.

Parrott, Benjamin B, Alicia Hudson, Regina Brady, and Cordula Schulz. 2012. “Control of Germline Stem Cell Division Frequency--a Novel, Developmentally Regulated Role for Epidermal Growth Factor Signaling.” *PloS One* 7 (5): e36460. doi:10.1371/journal.pone.0036460.

Scharf, Inon, Franziska Peter, and Oliver Y. Martin. 2013. “Reproductive Trade-Offs and Direct Costs for Males in Arthropods.” *Evolutionary Biology* 40 (2): 169–84. doi:10.1007/s11692-012-9213-4.

Schmidt, E. D., and A. Dorn. 2004. “Structural Polarity and Dynamics of Male Germline Stem Cells in the Milkweed Bug (Oncopeltus Fasciatus).” *Cell and Tissue Research* 318 (2): 383–94. doi:10.1007/s00441-004-0983-6.

Schmidt, Esther D., Elisabeth Sehn, and August Dorn. 2002. “Differentiation and Ultrastructure of the Spermatogonial Cyst Cells in the Milkweed Bug, Oncopeltus Fasciatus.” *Invertebrate Reproduction and Development* 42 (2–3): 163–78. doi:10.1080/07924259.2002.9652773.

Sharma, Rakesh, Ashok Agarwal, Vikram K Rohra, Mourad Assidi, Muhammad Abu-Elmagd, and Rola F Turki. 2015. “Effects of Increased Paternal Age on Sperm Quality, Reproductive Outcome and Associated Epigenetic Risks to Offspring.” *Reproductive Biology and Endocrinology* 13 (1): 35. doi:10.1186/s12958-015-0028-x.

Shuker, David M., and Leigh W. Simmons. 2014. “The Evolution of Insect Mating Systems.” In *The Evolution of Insect Mating Systems*. doi:10.1093/acprof:oso/9780199678020.003.0015.

Speakman, J. R. 2008. “The Physiological Costs of Reproduction in Small Mammals.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 363 (1490): 375–98. doi:10.1098/rstb.2007.2145.

Stearns, S C. 1992. “*The Evolution of Life Histories*.” *Zoology*. Vol. 1.

Vangompel, Michael J W, and Eugene Yujun Xu. 2011. “The Roles of the DAZ Family in Spermatogenesis: More than Just Translation?” *Spermatogenesis* 1 (1): 36–46. doi:10.4161/spmg.1.1.14559.

Wang, Lei, and D. Leanne Jones. 2011. “The Effects of Aging on Stem Cell Behavior in Drosophila.” *Experimental Gerontology* 46 (5). Elsevier Inc.: 340–44. doi:10.1016/j.exger.2010.10.005.

Wang, Lei, Catherine J. McLeod, and D. Leanne Jones. 2011. “Regulation of Adult Stem Cell Behavior by Nutrient Signaling.” *Cell Cycle* 10 (16): 2628–34. doi:10.4161/cc.10.16.17059.

Zajitschek, F., S R Zajitschek, C Canton, G Georgolopoulos, U Friberg, and A A Maklakov. 2016. “Evolution under Dietary Restriction Increases Male Reproductive Performance without Survival Cost.” *Proceedings.Biological Sciences / The Royal Society* 283 (1825): 10.1098/rspb.2015.2726. doi:10.1098/rspb.2015.2726 [doi].

Table 2.1. Repeated Measures ANOVA analysis of fertility and fecundity of females mated to milkweed-fed and sunflower-fed males at young and old ages.

TRAIT	FACTOR	F	d.f.	p-value
TOTAL EGGS PRODUCED	Between Subjects: Diet	0.931	1, 27	0.343
	Within Subjects: Age	15.346	1, 27	< 0.001
	Diet*Age	0.207	1, 27	0.652
PERCENT EGGS FERTILIZED	Between Subjects: Diet	13.481	1, 27	0.001
	Within Subjects: Age	3.304	1, 27	0.080
	Diet*Age	0.997	1, 27	0.327

Table 2.2. Two-way ANOVA analysis of cell cycle markers from older males fed milkweed or sunflower seeds.

TRAIT	FACTOR	F	d.f.	p-value
TOTAL SPERMATOCYSTS DIVIDING	Diet	0.001	1, 54	0.981
	Age	0.623	1, 54	0.433
	Diet x Age	0.274	1, 54	0.603
PHH3-POSITIVE SPERMATOCYSTS	Diet	4.942	1, 54	0.030
	Age	0.133	1, 54	0.716
	Diet x Age	1.354	1, 54	0.250
EdU-POSITIVE SPERMATOCYSTS	Diet	7.855	1, 54	0.007
	Age	0.580	1, 54	0.450
	Diet x Age	5.086	1, 54	0.028

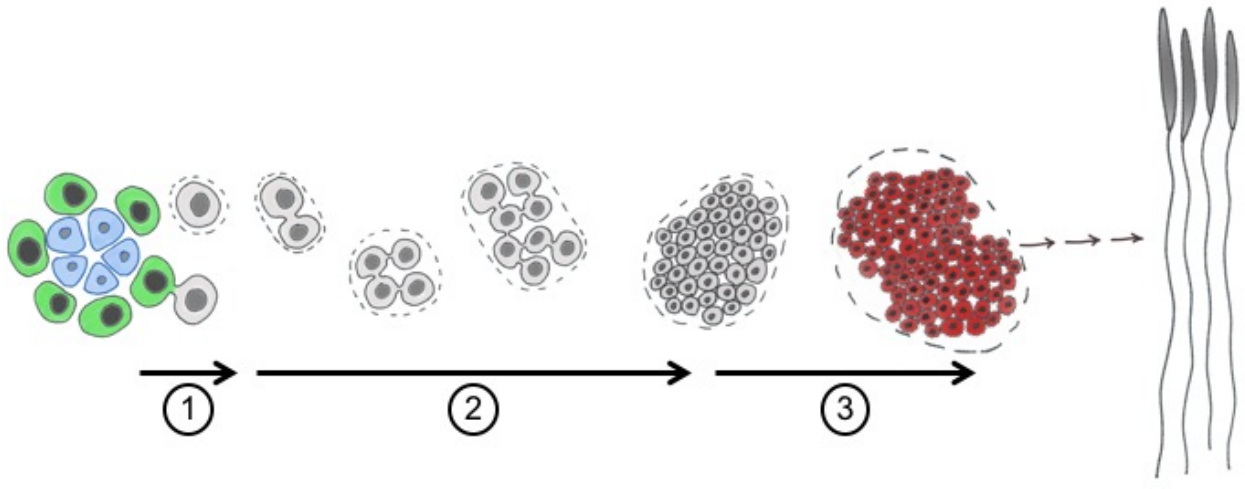

Figure 2.1. In *Oncopeltus fasciatus*, the germline stem cell niche is a rosette of cells at the tip of each testis tubule (blue cells; Schmidt et al. 2002). Germline stem cells (GSC; green cells) are in contact with the niche, which is essential to maintaining GSC identity. Spermatogenesis is initiated by a GSC dividing to produce one daughter cell that remains in the niche and remains a stem cell and another daughter cell that moves away from the niche and becomes a spermatogonial cell (Step 1; grey cells). Spermatogonial cells are encapsulated by cyst cells (dashed line) and undergo a series of mitotic transit divisions to form a 64 cell spermatocyst (Step 2; Ewen-Campen et al. 2013). The diploid spermatogonial cells then undergo meiosis to form the haploid spermatocytes (Step 3; red cells) that will differentiate into mature spermatids. Figure used with permission of Patricia J. Moore.

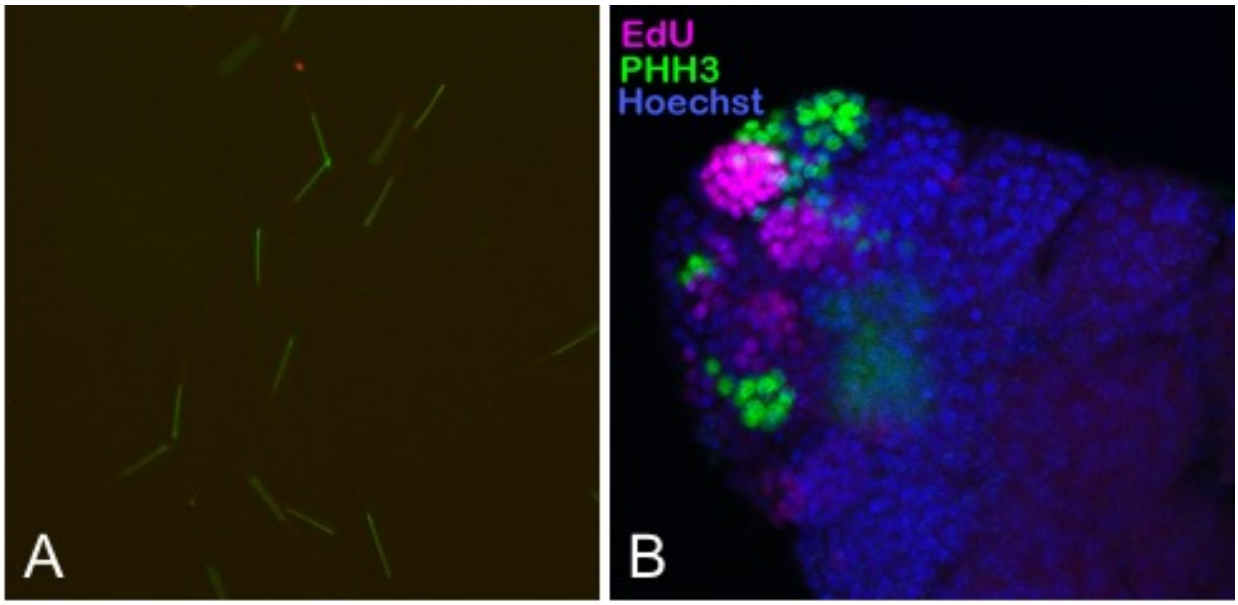

Figure 2.2. Example micrographs for analysis of sperm quality and transit amplification cell division cycles. (A) This photograph is a typical view of the sperm samples from the sperm viability experiment. Almost all sperm heads were stained green, indicating viability, with only the occasional sperm head stained red, indicating they were dead. (B) This photograph represents a typical staining pattern within the testiole showing spermatocysts stained for either the S-phase, incorporating EdU, or M-phase, stained with an antibody against a mitosis-specific histone modification, of the cell cycle. It is apparent that all spermatogonial cells within the spermatocyst are synchronized. All spermatogonial cells within a spermatocyst stain for the same stage within the cell cycle. For details, see the Materials & Methods.

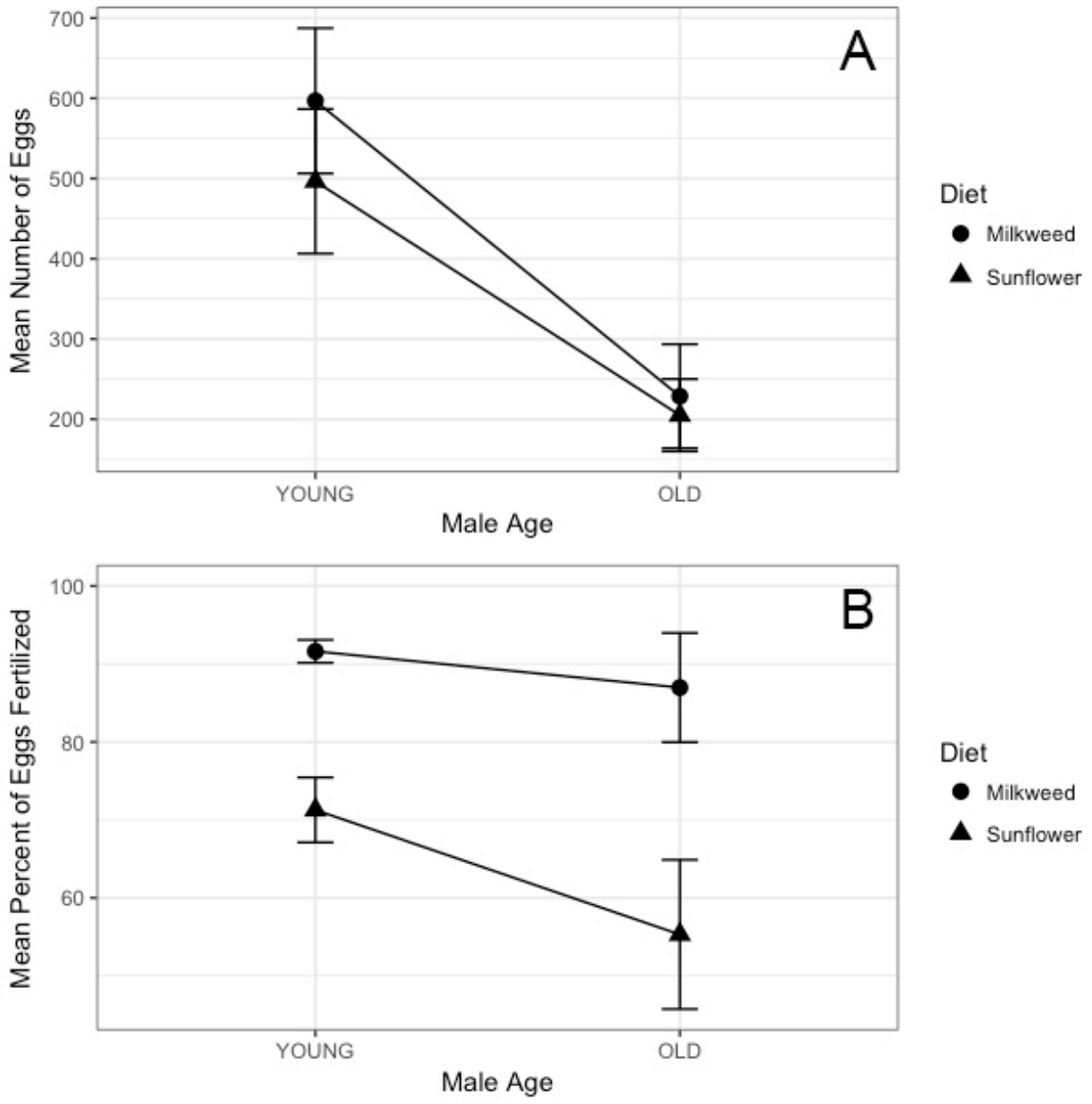

Figure 2.3. Male fertility and fecundity depends on age and diet. (A) The mean numbers of eggs laid by 7-10 day old females mated to males early in life is greater than the number of eggs laid by 7-10 day old females mated to the same male later in life. (B) Milkweed-fed males fertilized a higher proportion of eggs laid by their mates than sunflower-fed males at both ages. Error bars represent SE.

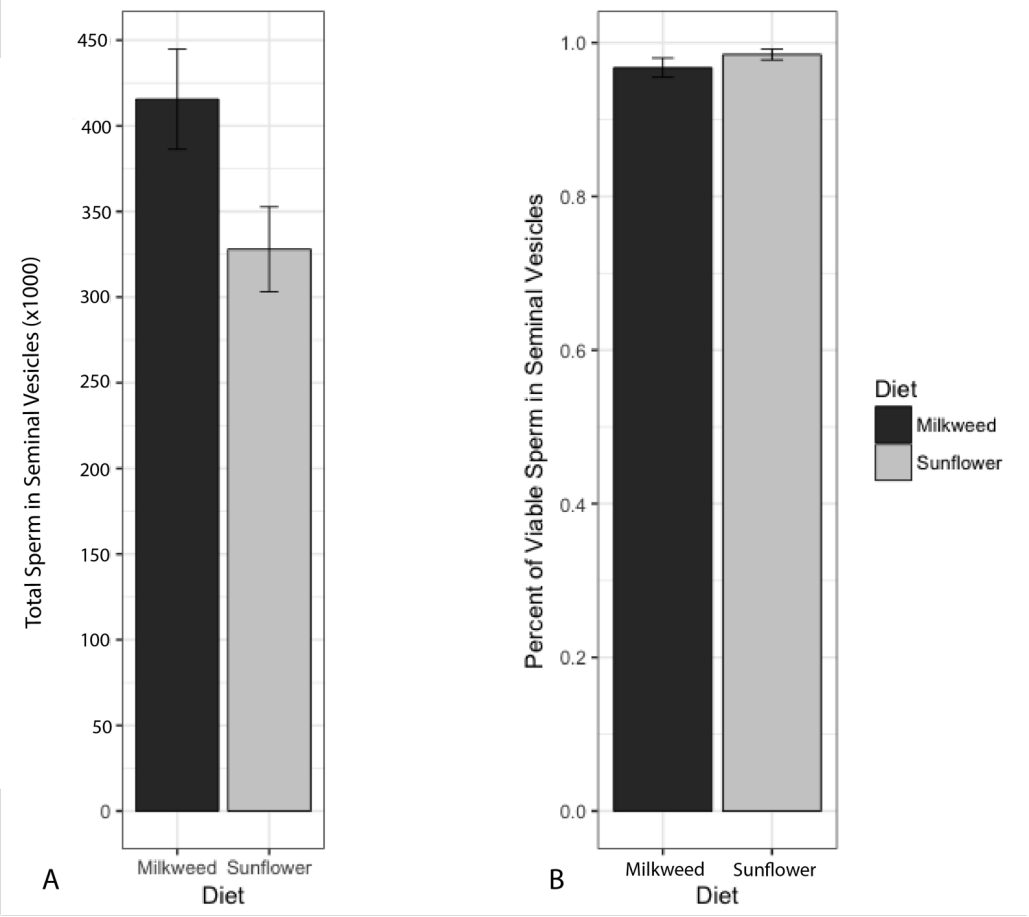

Figure 2.4. Diet affected sperm numbers, but not sperm viability. (A) Milkweed-fed males had more sperm in their seminal vesicles at 4-weeks post-adult eclosion than sunflower-fed males at the same age. (B) Males on both diets maintained high sperm viability and there was no difference with diet. Error bars represent SE.

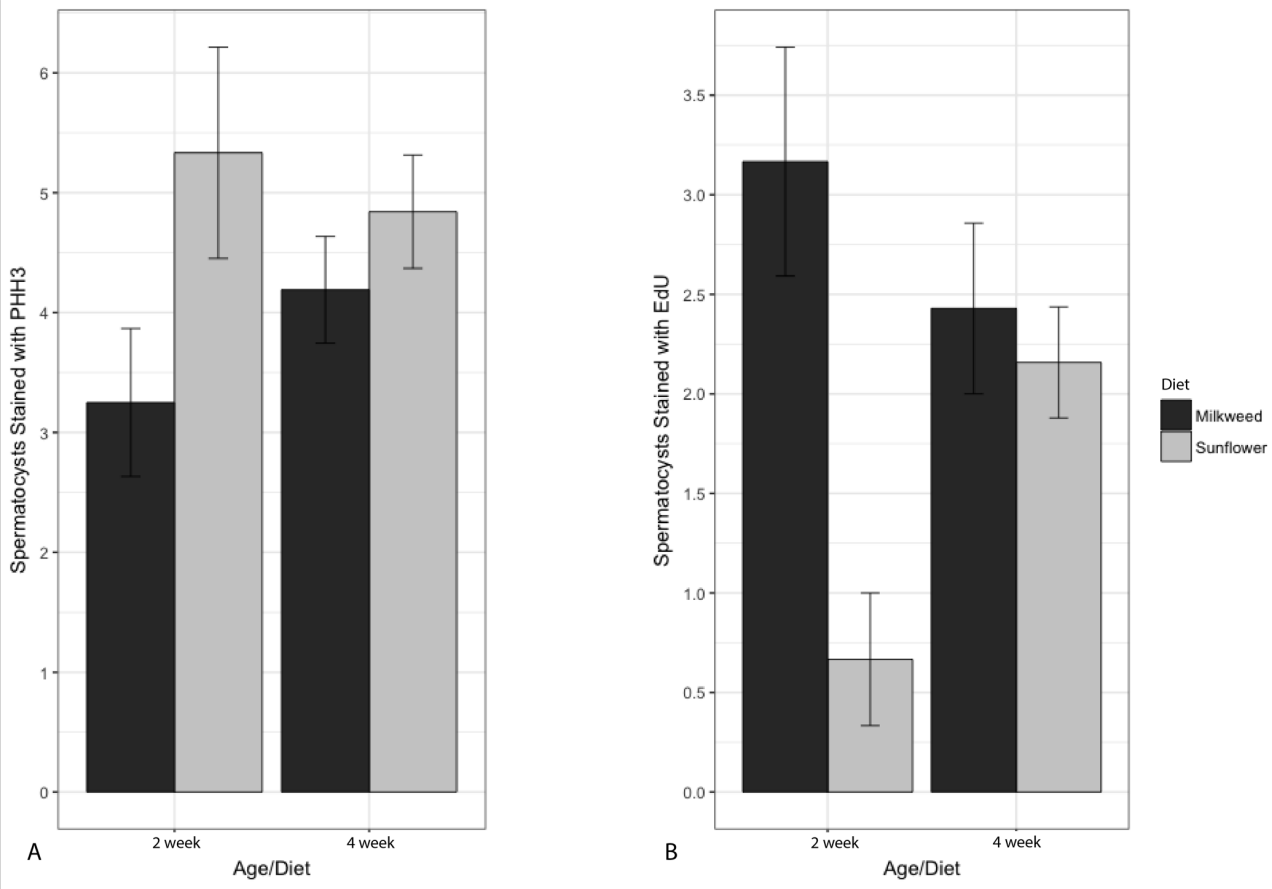

Figure 2.5. Diet, but not age, affect the progression of spermatogonial cells through the transit amplification division cell cycle. (A) Sunflower-fed males have more spermatocysts that stain for the M-phase of the cell cycle. (B) Milkweed-fed males have more spermatocysts that stain for the S-phase of the cell cycle, although the difference disappears over time. Error bars represent SE.

CHAPTER 3

GENOME-GUIDED TRANSCRIPTOME ANALYSIS OF MALE FERTILITY IN ONCOPELTUS FASCIATUS FED ANCESTRAL AND ADAPTED DIETS[1]

[1]A.E. Duxbury, G.R. Burke, and P.J. Moore. *To be submitted to G3: Genes, Genomes, Genetics.*

ABSTRACT

Oncopeltus fasciatus males that are fed different diets respond to the difference in environment by altering their reproduction rates and lifespan. Males that are fed milkweed seeds, the ancestral diet, are more likely to invest in reproduction at the expense of lifespan, while males fed sunflower seeds, the adapted diet, are more likely to invest less in reproduction and live longer. This study measured proximate genetic responses to these environmental changes by determining the number of differentially expressed genes between diets at the distal tip and proximal rest, and within diets at the distal tip and proximal rest. These comparisons can help us determine if there are changes between where the germline stem cells are being maintained and spermatogenesis is occurring within and between diet. We found that there were few differentially expressed genes between the diets, but the largest amount of differentially expressed genes were within the diets, between the distal tip and proximal rest of the testis. We also ran a gene set enrichment analysis in which we found that there could be a subset of genes associated with germline stem cells that are correlated with this expression data, and that they may be linked with diet and the change in life history trade-offs.

INTRODUCTION

A fundamental tenant of life history theory is that there is a trade-off between reproduction and lifespan. We expect that under favorable conditions organisms will invest in reproduction at the expense of somatic maintenance and survival. Under stressful or suboptimal conditions, however, individuals will invest in somatic maintenance and survival at the expense of reproduction (Flatt et al. 2013, Stearns 1989). Costs of reproduction are manifested by the trade-off between reproduction and somatic maintenance and have been documented across the tree of life and experimental selection experiments support an evolutionary trade-off. But a strong argument has been made that to truly understand this fundamental trade-off we need to understand the proximate mechanisms underlying the relationship between reproduction and somatic maintenance (Harshman & Zera 2007, Flatt & Heyland 2011). One model links reproduction and survival directly (Flatt 2011, Aguilaniu 2015, Maklakov & Immler 2016). Reproduction and somatic maintenance could be linked through competitive allocation of a limited pool of resources. Any environmental change, such as nutrient quality, that increases reproductive rate will decrease the energy available for somatic maintenance. While this model is appealing, the survival costs of reproduction are not inevitable and can be uncoupled. Alternatively, the observed trade-off could result because a common mediator regulates both traits with opposite effects on reproductive rate and survival. Nutrient quantity or quality may lead to a change, for example, in fat metabolism or stress response, which can have antagonistic effects on reproduction and lifespan. Data from *C. elegans* and *D. melanogaster* have provided some support for this model (e.g. Aguilaniu 2015).

Some of the complications in untangling the costs of reproduction may come because we still do not have good evidence about the mechanisms by which the cost of reproduction is incurred. When thinking about competitive allocation of a limited resource pool, researchers tend to focus on the large, energy expensive gametes of females. Recently it has been proposed, however, that the cost of maintaining the germline is a hidden cost of reproduction (Maklalov & Immler 2016) and that the rate of gamete production, and the control of germline stem cells, is a source of plasticity in the trade-off between reproduction and lifespan (Kaczmarczyk & Kopp 2011, Moore 2014). Therefore, it is essential that we examine the behavior of the germline stem cells and germline cells under variable environments with known life history consequences as a means of determining the mechanisms by which organisms plastically respond to different environments by adjusting life history decisions in response to current conditions and optimizing fitness.

Plasticity in life history strategies in the milkweed bug Oncopeltus fasciatus

Wild populations of the milkweed bug, *Oncopeltus fasciatus*, feed on seeds of milkweed (*Asclepias spp*.), but the population we study has been in culture for over 40 years and have evolved to utilize either sunflower or milkweed seeds as a food source (Moore & Attisano 2011, Newcombe et al. 2015a, 2015b). Thus, this population offers the opportunity to study life history evolution under variable nutritional environments in a single population. Females show no difference in fitness on the sunflower and milkweed diets (Moore & Attisano 2011). Males however, while having equal lifetime reproductive success, demonstrate different patterns of life history trade-offs on the two diets (Attisano et al. 2012). In this experiment, all females mated to experimental males were 7 days post-eclosion and fed the adapted diet of sunflower

seeds. There was no effect of male diet on female fecundity. There is, however, an effect of male diet on male fertility and lifespan. Male milkweed bugs fed the adapted diet of sunflower live longer than males fed milkweed, but demonstrate an age-related loss of fertility. Males fed milkweed maintain late life fertility, but pay the cost in lifespan, surviving a maximum of 60 days in comparison to over 90 days for the sunflower-fed males. Diet-induced plasticity in reproductive life history is not unique to milkweed bugs. Recently it has been shown that the nematode *Caenorhabditis elegans* fed its preferred diet of *E. coli* strain HB101 has increased early reproduction and more rapid onset of reproductive aging than worms fed the strain OP50 (Sowa et al. 2015).

In addition to these key life history studies, *Oncopeltus* has been used as a model in evolutionary developmental biology studies and key developmental genes have been identified. A molecular 'toolbox' has been developed for *Oncopeltus*, including a sequenced genome (Richards & Murali 2015). Crucially for our study, the male reproductive system, including morphological identification of the germline stem cells and the associated niche, is well characterized and genes associated with the germline have been identified (Ewen-Campen et al. 2013).

To examine proximate genetic mechanisms that underlie this variation in life history pattern, we measured whole genome transcriptional responses from the testes of males fed sunflower and those fed milkweed. We collected our samples and analyzed our transcriptome data with three distinct goals. First, we were interested in differentiating between transcriptional differences associated with the different biological activities that take place in the different regions of the testes (Figure 3.1). At the tip of the testis is the region that contains

the germline stem cells. Germline stem cells are germline cells that reside in a niche and divide to produce one daughter cell that remains in the niche and remains a stem cell, thus providing a reservoir of cells for future reproduction, and a second daughter cell that moves out of the niche and develops into a germ cell. These diploid spermatogonial cells are encapsulated by cyst cells and undergo a series of mitotic transit amplification divisions to form a 64 cell spermatocyst. The spermatocygonial cells then undergo meiosis to form the haploid spermatocytes that will differentiate into mature spermatids. We compared the transcriptional profiles between the testis distal tip and proximal end of the testes to search for genes associated with either control of the germline, which we predicted would be enriched in the testis distal tip transcriptomes, or spermatogenesis, which would be enriched in the proximal end of the testes.

We then tested the hypothesis that the variation in fertility we see in older males on the two diets arose due to variation in the rate of division of either the germline stem cells to produce spermatogonial cells or in the rate or number of transit amplification divisions by comparing the transcriptional profiles of the testis tips of milkweed and sunflower-fed males. We predicted that we would see significant differences between diets in this region of the testes. We also predicted that there would be fewer differences in the transcriptional profiles among the two diets in the distal region of the testes as we had no reason to predict that diet would affect the process of spermatogenesis.

METHODS

Insect Rearing

Large milkweed bugs, *O. fasciatus*, were obtained from Carolina Biological Supply (Burlington, NC, USA) where bugs have been raised for hundreds of generations. All colonies and individuals were kept at 26°C and 16:8h light:dark. Eggs were collected from mass colonies and left to mature through 5th instar in a nymphal colony with sunflower seeds and water. When adults emerged, males were put into individual dishes with either sunflower or milkweed seeds and water and females were put into colonies with sunflower seeds and water by date emerged. Males were then mated with a virgin female at 2 weeks, a female from the mass colony at 3 weeks and again with a virgin female at 4 weeks. Each mating took place over 48 hours to allow for copulation to occur. At the end of the last mating, the male's testes were dissected out and placed in RNAlater (Ambion, Waltham, MA, USA) and stored at -20°C until all samples were ready for RNA extraction. 20 males were used for each dietary condition.

RNA extraction

Testes were separated into two regions, the testis tip, cut about 2mm from the apex of the testis with a sterile disposable microscalpel, containing the germline stem cells and the niche, and the distal testis (Figure 1). Total RNA was extracted from each testis region using RNeasy Micro RNA extraction kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The testes of a single male were sufficient to obtain a minimum of 50ng of RNA from both regions. The RNA was quantified by measuring the absorbance at 260 nm using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). A 1% agarose gel electrophoresis confirmed the integrity of the RNA while the purity was assessed by an Agilent 2100

Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). There were a total of 8 biological replicates per sample (milkweed-fed distal tip, milkweed-fed proximal rest, sunflower-fed distal tip, and sunflower-fed proximal rest).

RNA sequencing

TruSeq stranded library preparation was completed and Paired-end Illumina sequencing was performed on an Illumina HiSeq 2500 system (100 bp paired-end sequencing) at the Georgia Genomics Facility, University of Georgia, Athens, GA, USA.

RNA quality

In total, there were 88,728,705 raw RNA reads from the Illumina runs, and after trimming and cleaning using FastX-toolkit there were 77,200,923 reads that were at least at a 30 Phred score for 75% of the bases, determined using FastQC (Andrews 2010); the low-quality sequences and barcodes were then removed using FASTX-toolkit (Hannon 2009). These reads were subjected to further analysis in TopHat and Cufflinks (Trapnell et al. 2009, 2012).

Annotation

Cleaned reads were aligned to the *O. fasciatus* genome (Hughes et al. 2015) using TopHat (Trapnell et al. 2009). These aligned reads were then assembled and differentially expressed genes were determined using the Cufflinks package: Cufflinks, Cuffcompare, Cuffmerge, and Cuffdiff. Each sample was assembled through Cufflinks and compared to the annotation through Cuffcompare separately before being merged together with Cuffmerge. Cuffdiff was then used on the merged assembly to find differentially expressed genes and transcripts. CummeRbund was used to plot FPKM expression results (Trapnell et al. 2012).

Quantitative PCR

To validate the results of the RNA-seq data, quantitative PCR was performed on RNA isolated from two whole Oncopeltus fasciatus testes dissected from males that were treated to the same conditions as the males dissected for transcriptomic studies. Two housekeeping genes, *actin* and *GAPDH*, and six genes that play a role in spermatogenesis or germline stem cell maintenance, *boule*, *vasa*, *tektin-1*, *krueppel-like factor*, *hsp83*, *corazonin precursor* and *rp L35Ae* were chosen for qPCR study based on their expression profiles in the transcriptome comparison (see Results) and information about their functions from other studies (see Discussion). Four replicates per RNA sample were run using SYBR Green Master Mix using a Rotor-Gene Q (Qiagen) and data was analyzed using the $2^{-\Delta CT}$ method (Livak & Schmittgen 2001). For this method, because it is relative quantitation, there is an assumption that the amplification efficiency is at 100%, or 1 (Rao et al. 2013). Forward and reverse primers used for each run can be found in Table 3.1.

RESULTS

To answer our three main questions, we looked at the number of genes that were different between each of the two diets, sunflower and milkweed seeds, and the genes that were upregulated in the distal tip of the testis versus the proximal rest of the testis. We also determined the number of expressed genes in each of the samples, sunflower distal tip and proximal rest and milkweed distal tip and proximal rest, and compared the testis regions to each other, between and within diets.

Of the 65,535 genes returned through annotation with Cufflinks, 3,660 were significantly expressed, with a p-value less than 0.05. Comparing the diets to each other,

sunflower-fed males had 608 upregulated genes, and milkweed had 611 upregulated genes. The two diets shared 467 of these upregulated genes (Figure 3.2A). Downregulation occurs in 4,310 of genes found in sunflower-fed males, 2,219 milkweed-fed male genes, and these two diets share 2,137 downregulated genes (Figure 3.2B).

To see if there is a difference between the distal tip and proximal rest of the testis within and between diets, which could mean a difference in germline maintenance and turnover, as well as other processes, we determined the number of upregulated and downregulated genes from our set of significantly expressed genes. We completed this for each portion of the testis, distal tip and proximal rest, based on the diet of the male, sunflower or milkweed seeds. In the distal tip subset, we found 2,124 genes to be upregulated in sunflower distal tip, 62 genes upregulated in milkweed-fed males' distal tip, and 2,134 genes that were upregulated in both diets' distal testis tips.

Investigating the difference in the proximal rest portion of the testis, and therefore the portion of the testis that produces sperm, we looked at the number of up- and downregulated genes in the proximal rest portion of the testis. We determined that there are 3 genes upregulated in milkweed-fed proximal rest testis, and 607 genes shared between both diets sunflower and milkweed seeds, where sunflower-fed males do not have any genes that are upregulated in the proximal rest area of their testis.

Quantitative PCR

Genes that we thought were interesting based on the literature (Ewen-Campen et al. 2013, Harvanek et al. 2017) and that we found in our significantly expressed group of

transcripts were then tested with qPCR to determine expression level. We used sunflower-fed males as our reference sample and milkweed-fed males as our target sample.

Of the six genes validated with qPCR, many followed the patterns seen in the transcriptome data. *Tektin-1*, *boule* (Figure 3.3), *rpL35Ae* (Figure 3.5), *hsp83*, *corazonin precursor* (Figure 3.6), and *vasa* (Figure 3.4) are more abundant in the testis distal tip than in the proximal rest in the transcriptome, but we cannot clearly say that this is truly the case in the qPCR data because we used whole testis. *Krueppel-like factor* has a small, but not significant, fold change in the up-regulated direction (Figure 3.7); however, it is not significant one way or another in either the transcriptome or the qPCR.

DISCUSSION

The whole genome transcriptional responses from the testes of males fed sunflower seeds and those fed milkweed seeds were examined to determine the proximate genetic mechanisms that underlie the variation that is seen in life history. We predicted that there would be a difference in the transcriptional profiles between the two diets' whole testes, within the testis themselves, i.e. the distal tip and proximal rest, and between diets and the two different sections, distal tip and proximal rest. We expected that there would be more differentially expressed genes in the distal tips of both diets than in the proximal rest because of the nature of what is occurring in that area. The distal tips of the testes have multiple cell types and are going through multiple rounds of division, while the proximal rest of the testes are converting spermatocysts into sperm, and that does not require as many different pathways involved (Matunis et al. 2012, Yang et al. 2013, Zhang et al. 2013, Ewen-Campen et al. 2013).

Overall, there was a low number of differentially expressed genes (DEGs) in this transcriptome. This is could be possible because of the similarities in the mechanisms that create and/or regulate sperm production in all organisms; these genes are conserved throughout most species (reviewed in Matunis et al. 2012).

To examine our first set of predictions, we looked at the genes that were either significant and up- or downregulated, or differentially expressed in these specific areas. As milkweed seeds are the ancestral diet, there may be more genes differentially expressed in the germline stem cells because this state is the normal state *of O. fasciatus* in the wild. Sunflower seeds are the diet in which *O. fasciatus* has been adapted to live on in the lab, but is also an adapted diet, so the organisms may have a different mechanism of spermatogenesis or turnover of GSCs, because the males do in fact live longer than the milkweed fed males and therefore do not put as many resources into reproduction (Kaczmarczyk & Kopp 2011, Moore 2014).

We came across specific genes such as, *boule*, *vasa*, *piwi* (Ewen-Campen et al. 2013), *ribosomal protein L35Ae* (Hasygar & Hietakangas 2014), and *corazonin precursor* (Veenstra 2009, Havarnek et al. 2017). These genes have roles in either spermatogenesis or GSC maintenance and/or division (Matunis et al. 2012). Each fell in line with the prediction that testis tip transcriptome would be enriched in genes associated with germline stem cell maintenance and/or division, and the transcripts from the distal testis region would be associated with spermatogenesis. However, when the diets of milkweed-fed and sunflower-fed males were compared, there were more differentially expressed genes between the diets than was found among the different regions of the testis. Between the diets, there could be a

difference in the amount of regulation of spermatogenesis, turnover of germline stem cells, or the males could not be getting as nutritious of a meal. These factors can lead to one diet then having more expression of genes that regulate spermatogenesis. More specifically, the milkweed seed diet may be one in which this occurs. It has been shown that milkweed seed fed males store more sperm in their seminal vesicles than sunflower fed males (Duxbury et al. 2017), and this could be because of these DEGs. We could use this list of genes used for the GSEA to expand our search in the DEGs to determine if any of the differentially expressed genes in the transcriptome plays a necessary role in the regulation of spermatogenesis.

Boule was found to be upregulated in the testis tip, and *boule* is necessary for entry into spermatogenesis, the switch from mitosis to meiosis (Sekiné et al. 2015). *Boule* is transcribed exclusively in the testis, in spermatocytes and is required for germ cell specification (Fuller 1998). The switch from mitosis to meiosis is when all the spermatocysts are still joined together and are dividing and create a large interconnected spermatocyst that then undergoes mitosis, which is when the cells would then be in the spermatogenesis process (Figure 1). Knowing that *boule* is exclusive to the testis and is needed for germ cell specification, we can use the up-regulation in the tip as a sign that we have found *boule* and that even though it may not be specific to a diet, maybe slightly higher in sunflower-fed males, it is regulating spermatogenesis, but through this switch to meiosis, which we may have caught, as our methods for dissection of the distal tip were not very precise (Figure 3.1).

Vasa is also found to be differentially regulated in the tips of the testis versus the rest. This corresponds with the literature on *vasa*, in that it is a key player in the germline of many organisms. *Vasa* is conserved in germ line development, and that includes germ cell

specification, proliferation, and maintenance (Gustafson & Wessel 2010). *Vasa* is also required to maintain cyst synchrony in spermatogenesis, and it may also be required for the secondary spermatogonial cell to enter the correct progression as they become spermatocytes (Ewen-Campen et al. 2013). Therefore, *vasa* and *boule* could both possibly be under the control of a factor that is linked to the diet. Thus, the two could be controlled by lipid or protein metabolism in some manner.

While not many studies have been conducted on *ribosomal protein L35Ae* (*rp L35Ae*), it has been shown in *D. melanogaster* that the long ribosomal protein may play a role in the insulin signaling pathway and can affect the growth of an organism (Hasygar & Hietakangas 2014), which could in turn affect longevity and reproduction. This pathway, the insulin signaling pathway, is important for insects in that it controls growth and reproduction. *RP L35Ae* was found to be more highly expressed in the testis tip than the distal testis, but it was also more highly expressed in the sunflower-fed males. With this being differentially expressed between the two diets, it may lead to the thought that sunflower-fed males can activate the insulin pathway more easily and therefore possibly activate further downstream pathways that maintain GSCs or create sperm. Evidence in *D. melanogaster* suggests that there is a connection between the gonad, 20-hydroxyecdysone (20E), the insulin/insulin-like (IIS) pathway, and longevity. Germline-less, long-lived flies have an increased expression of an insulin binding protein (an antagonist) that is induced by 20E (Gáliková et al. 2011). Therefore, the sunflower diet possibly activating the IIS pathway is a way for the males to control the amount of sperm made and to live longer.

One interesting find is *corazonin precursor*. This neuropeptide was found to be differentially upregulated in the testis distal tips, in both milkweed and sunflower. Corazonin is a neuropeptide that is normally found in the corpora cardiaca of many insects, e.g. the fruit fly, walking sticks, locusts, and was also found in the subesophaegeal and thoracic ganglia of *Triatoma infestans*, a Heteropteran, which means it can be classified as a neurohormone (Settembrini et al. 2011). This neurohormone has been found to mediate the effect of mating on lifespan. Corazonin may play a role further down in GSC regulation by acting upon a regulator of GSC maintenance *FOXO* (Harvanek et al. 2017). Therefore, *corazonin precursor* could be part of a pathway that is in fact mediating GSC maintenance based on the mating rate of an individual.

Piwi is found in the GSCs and the cyst stem cells, and is useful for maintenance and differentiation, however, it is not necessary in the germline stem cells. *Piwi* is known to interact with vasa and plays a role in silencing transposable elements in the germline (Ewen-Campen et al. 2010). As *piwi* is found in the transcriptome of the testis tip, but more so in the sunflower tip, as well as in immunofluorescent studies (Gonzalez et al. 2015), it could possibly play a role in the cells that nourish the GSCs before they enter spermatogenesis. Therefore, *piwi* may be helpful in the creation of spermatocysts. This may have to do with the diet as sunflower males do tend to invest in fertility over their whole lifespan, rather than at one age like the milkweed fed males (Attisano et al. 2012). Although not validated with qPCR, this is still an interesting find in our study.

Our results did not document large numbers of differentially expressed genes among our treatments, despite the differences in phenotype among males on the two diets (Attisano

et al. 2012, Duxbury et al. 2017). We predicted that the increase in fertility of males, which results from increased sperm numbers, would result from an increase in GSC turnover and thus more spermatogonial cells being 'born'. We further predicted that this difference would be reflected by differential expression of genes involved in GSC turnover. Our transcriptome analysis tested for differences in expression of individual genes associated with control of germline stem cell behavior in the testis distal tip. One potential explanation for this is that differences in phenotype are primarily controlled at another step in the process of spermatogenesis.

Even though there was a low number of significant genes, there was valuable information learned from the transcriptome of these insects' testis. Many of the genes thought to be differentially expressed, were based on the distal tip of the testis versus the proximal rest of the testis, even if they were not separated by diet. However, we did still see some differences in diet which can lead us to further insight into how diet can affect the amount of sperm a male can produce. A diet higher in polyunsaturated fatty acids (PUFAs), are usually more disadvantageous (Bustos and Partridge 2017), in that organisms that live longer have a higher monounsaturated fatty acid:polyunsaturated fatty acid ratio. Sunflower-fed males have a composition of more stearic acid, a saturated fatty acid, and linoleic acid, a PUFA, than milkweed fed males, which have more oleic acid, a monounsaturated fatty acid (Nation and Bowers 1982). These differences in fatty acid compositions, just this slightly altered ratio, in the bugs after eating milkweed and sunflower seeds could be a reason in which the regulation is slightly different. Being that insulin-signaling controls some of GSC division and the pathways for maintenance (McLeod et al. 2010), it could be that a slight change in diet composition could

explain this plasticity in life history. It could be possible that some of the DEGs that we have pulled out of this transcriptome could be the link between this trade-off in diet, longevity and reproduction.

REFERENCES

Aguilaniu, Hugo. 2015. "The Mysterious Relationship between Reproduction and Longevity." *Worm* 4 (2): e1020276. doi:10.1080/21624054.2015.1020276.

Attisano, A., A. J. Moore, and P. J. Moore. 2012. "Reproduction-Longevity Trade-Offs Reflect Diet, Not Adaptation." *Journal of Evolutionary Biology* 25 (5): 873–80. doi:10.1111/j.1420-9101.2012.02476.x.

Au, C. E., L. Hermo, E. Byrne, J. Smirle, A. Fazel, R. E. Kearney, C. E. Smith, et al. 2015. "Compartmentalization of Membrane Trafficking, Glucose Transport, Glycolysis, Actin, Tubulin and the Proteasome in the Cytoplasmic droplet/Hermes Body of Epididymal Sperm." *Open Biology* 5 (8): 150080. doi:10.1098/rsob.150080.

Andrews S. 2010. "FastQC: a quality control tool for high throughput sequence data." Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Duxbury, A.E., B. Weathersby, Z. Sanchez, & P. J. Moore. 2017. "A study of the transit amplification divisions during spermatogenesis in *Oncopeltus fasciatus* to assess plasticity in sperm numbers or sperm viability under different diets." Manuscript submitted for publication.

Ewen-Campen, B., N. Shaner, K. A. Panfilio, Y. Suzuki, S. Roth, and C. G. Extavour. 2011. "The Maternal and Early Embryonic Transcriptome of the Milkweed Bug Oncopeltus

Fasciatus." *BMC Genomics* 12 (1). BioMed Central Ltd: 61. doi:10.1186/1471-2164-12-61.

Ewen-Campen, B., Jones, T.E.M. & Extavour, C.G., 2013. Evidence against a germ plasm in the milkweed bug Oncopeltus fasciatus, a hemimetabolous insect. Biology Open, 2, pp.556–568.

Flatt, T. & Heyland, A., 2011. "Mechanisms of life history evolution."

Francischetti, I. M. B., A. H. Lopes, F. A. Dias, V. M. Pham, and J. M. C. Ribeiro. 2007. "An Insight into the Sialotranscriptome of the Seed-Feeding Bug, Oncopeltus Fasciatus." *Insect Biochemistry and Molecular Biology* 37 (9): 903–10. doi:10.1016/j.ibmb.2007.04.007.

Fuller, M T. 1998. "Genetic Control of Cell Proliferation and Differentiation in Drosophila Spermatogenesis." *Seminars in Cell & Developmental Biology* 9 (4): 433–44. doi:10.1006/scdb.1998.0227.

Gáliková, Martina, Peter Klepsatel, Gabriele Senti, and Thomas Flatt. 2011. "Steroid Hormone Regulation of C. Elegans and Drosophila Aging and Life History." *Experimental Gerontology* 46 (2–3): 141–47. doi:10.1016/j.exger.2010.08.021.

Gonzalez, Jacob, Hongying Qi, Na Liu, and Haifan Lin. 2015. "Piwi Is a Key Regulator of Both Somatic and Germline Stem Cells in the Drosophila Testis." *Cell Reports* 12 (1). The Authors: 150–61. doi:10.1016/j.celrep.2015.06.004.

Gustafon, E.A., and G.M. Wessel. 2011. "Vasa Genes: Emerging Roles in the Germ Line and Multipotent Cells." *Bioessays* 32 (7): 626–37. doi:10.1002/bies.201000001.Vasa.

Hannon, G. 2009. "FASTX-toolkit: FASTQ/A short-reads pre-processing tools." Available online at: http://hannonlab.cshl.edu/fastx_toolkit/index.html

Harshman, Lawrence G., and Anthony J. Zera. 2007. “The Cost of Reproduction: The Devil in the Details.” *Trends in Ecology and Evolution*. doi:10.1016/j.tree.2006.10.008.

Harvanek, Zachary M., Yang Lyu, Christi M. Gendron, Jacob C. Johnson, Shu Kondo, Daniel E. L. Promislow, and Scott D. Pletcher. 2017. “Perceptive Costs of Reproduction Drive Ageing and Physiology in Male Drosophila.” *Nature Ecology & Evolution* 1 (6): 152. doi:10.1038/s41559-017-0152.

Hasygar, Kiran, & Ville Hietakangas. 2014. “p53- and ERK7-Dependent Ribosome Surveillance Response Regulates Drosophila Insulin-Like Peptide Secretion.” *PLoS Genetics* 10 (11). doi:10.1371/journal.pgen.1004764.

Hsu, H.-J. & D. Drummond-Barbosa. 2009. “Insulin levels control female germline stem cell maintenance via the niche in Drosophila.” *PNAS* 106(4), pp.1117–1121.

Hughes, D. S. T., S. Koelzer, K. A. Panfilio and S. Richards. 2015. “Oncopeltus fasciatus genome annotations v0.5.3.” *Ag Data Commons.*

Kaczmarczyk, A.N. & Kopp, A., 2011. “Germline stem cell maintenance as a proximate mechanism of life-history trade-offs?” *BioEssays: news and reviews in molecular, cellular and developmental biology* 33(1), pp.5–12.

Li, Y., Q. Ma, C. M. Cherry, and E. L. Matunis. 2014. “Steroid Signaling Promotes Stem Cell Maintenance in the Drosophila Maintenance.” *Developmental Biology* 394: 129–41.

Livak, K. J. & T. D. Schmittgen (2001). “Analysis of relative gene expression data using real-time quantitative PCR and the 2-delta delta Ct method.” *Methods* 25:402-408.

Lynn, D. A., H. M. Dalton, J. N. Sowa, M. C. Wang, A. A. Soukas, and S. P. Curran. 2015. “Omega-3 and -6 Fatty Acids Allocate Somatic and Germline Lipids to Ensure Fitness during

Nutrient and Oxidative Stress in Caenorhabditis Elegans." *PNAS* 112 (50). doi:10.1073/pnas.1514012112.

Matunis, E. L., R. R. Stine, and M. de Cuevas. 2012. "Recent Advances in Drosophila Male Germline Stem Cell Biology." *Spermatogenesis* 2 (3): 137–44. doi:10.4161/spmg.21763.

Maklakov, A. A., and S. Immler. 2016. "The Expensive Germline and the Evolution of Ageing." *Current Biology*. doi:10.1016/j.cub.2016.04.012.

McLeod, C. J., L. Wang, C. Wong, and D. L. Jones. 2010. "Stem Cell Dynamics in Response to Nutrient Availability." *Current Biology* 20: 2100–2105.

Moore, P.J. 2014. "Reproductive Physiology and Behaviour." *The Evolution of Insect Mating Systems* 1–24.

Nation, J.L. & W.S. Bowers. 1982. "Fatty acid composition of milkweed bugs Oncopeltus fasciatus and the influence of diet." *Insect Biochemistry* 12(4): 455–459.

Newcombe, D., J. Hunt, C. Mitchell, and A. J. Moore. 2015. "Maternal Effects and Maternal Selection Arising from Variation in Allocation of Free Amino Acid to Eggs." *Ecology and Evolution* 5 (12): 2397–2410. doi:10.1002/ece3.1524.

Newcombe, D., P. J. Moore, and A. J. Moore. 2015. "The Role of Maternal Effects in Adaptation to Different Diets." *Biological Journal of the Linnaean Society* 114 (1): 202–11. doi:10.1111/bij.12408.

Pierson, S. N., J. N. Butler and R. G. Foster. 2003. "Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis." *Nucleic Acids Research* 31(14).

Rao, Xiayu et al. 2013. “An Improvement of the 2ˆ(–delta Delta CT) Method for Quantitative Real-Time Polymerase Chain Reaction Data Analysis.” *Biostatistics, bioinformatics and biomathematics* 3.3:71–85.

Richards, Stephen. 2017. “Milkweed Bug Genome Project.” https://www.hgsc.bcm.edu/arthropods/milkweed-bug-genome-project.

Richards, Stephen, and Shwetha C. Murali. 2015. “Best Practices in Insect Genome Sequencing: What Works and What Doesn’t.” *Current Opinion in Insect Science* 7: 1–7. doi:10.1016/j.cois.2015.02.013.

Schmidt, E.D. & A. Dorn, 2004. “Structural polarity and dynamics of male germline stem cells in the milkweed bug (Oncopeltus fasciatus).” *Cell and Tissue Research* 318:383–394.

Sekiné, K., T. Furusawa, & M. Hatakeyama, 2015. “The boule gene is essential for spermatogenesis of haploid insect male.” *Developmental Biology* 399(1):154–163.

Settembrini, B. P., D. De Pasquale, M. Postal, P. M. Pinto, C. R. Carlini, and M. J. Villar. 2011. “Distribution and Characterization of Corazonin in the Central Nervous System of Triatoma Infestans (Insecta: Heteroptera).” *Peptides* 32:461–68. doi:10.1016/j.peptides.2010.10.020.

Sowa, J. N., A. S. Mutlu, Fan Xia, and Meng C Wang. 2015. “Olfaction Modulates Reproductive Plasticity through Neuroendocrine Signaling in Caenorhabditis Elegans.” *Current Biology* 25 (17). Elsevier Ltd: 2284–89. doi:10.1016/j.cub.2015.07.023.

Stearns, S.C. 1989. “Trade-Offs in Life History Evolution.” *Functional Ecology* 3 (3): 259–68.

Trapnell, C., L. Pachter & S. L. Salzberg. 2009. “TopHat: discovering splice junctions with RNA-Seq.” *Bioinformatics* 25(9):1105-1111.

Trapnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L. Salzberg, J. L. Rinn and L. Patcher. (2012). “Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks.” *Nature Protocols* 7(3):562-578.

Veenstra, Jan A. 2009. “Does Corazonin Signal Nutritional Stress in Insects?” *Insect Biochemistry and Molecular Biology*. doi:10.1016/j.ibmb.2009.09.008.

Yang, Sheng An, Wen Der Wang, Ciao Ting Chen, Chen Yuan Tseng, Yi Ning Chen, and Hwei Jan Hsu. 2013. “FOXO/Fringe Is Necessary for Maintenance of the Germline Stem Cell Niche in Response to Insulin Insufficiency.” Developmental Biology 382 (1): 124–35. doi:10.1016/j.ydbio.2013.07.018.

Zhang, Zhao, Xiangdong Lv, Jin Jiang, Lei Zhang, and Yun Zhao. 2013. “Dual Roles of Hh Signaling in the Regulation of Somatic Stem Cell Self-Renewal and Germline Stem Cell Maintenance in Drosophila Testis.” *Cell Research* 23: 1–4. doi:10.1038/cr.2013.29.

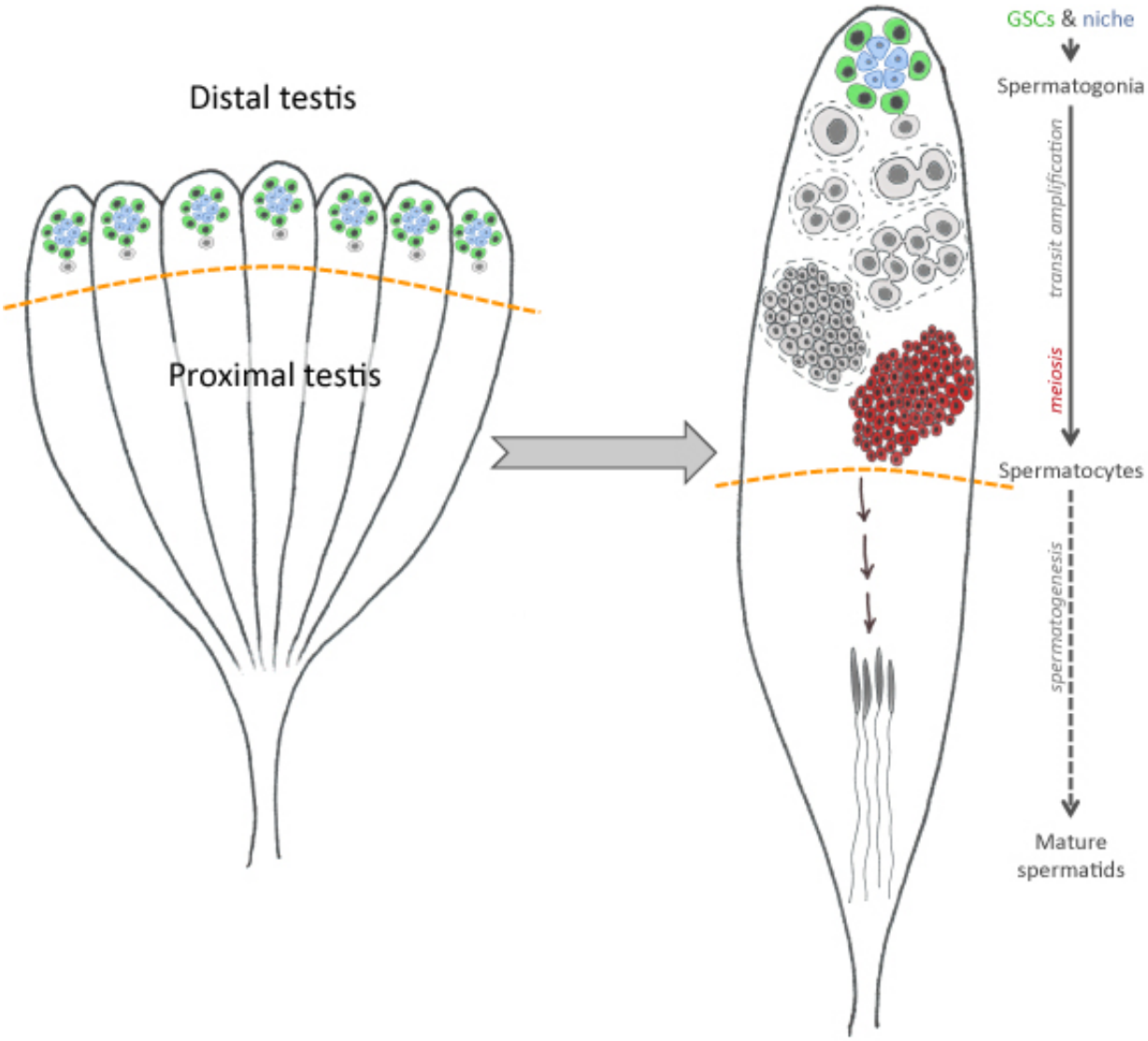

Figure 3.1. Image of *O. fasciatus* testis showing approximate cutoff for "Distal" and "Proximal" of testis. The cut was made about 2mm from the apex of the testis to collect the germline stem cells and germline niche. Figure used with permission of Patricia J. Moore.

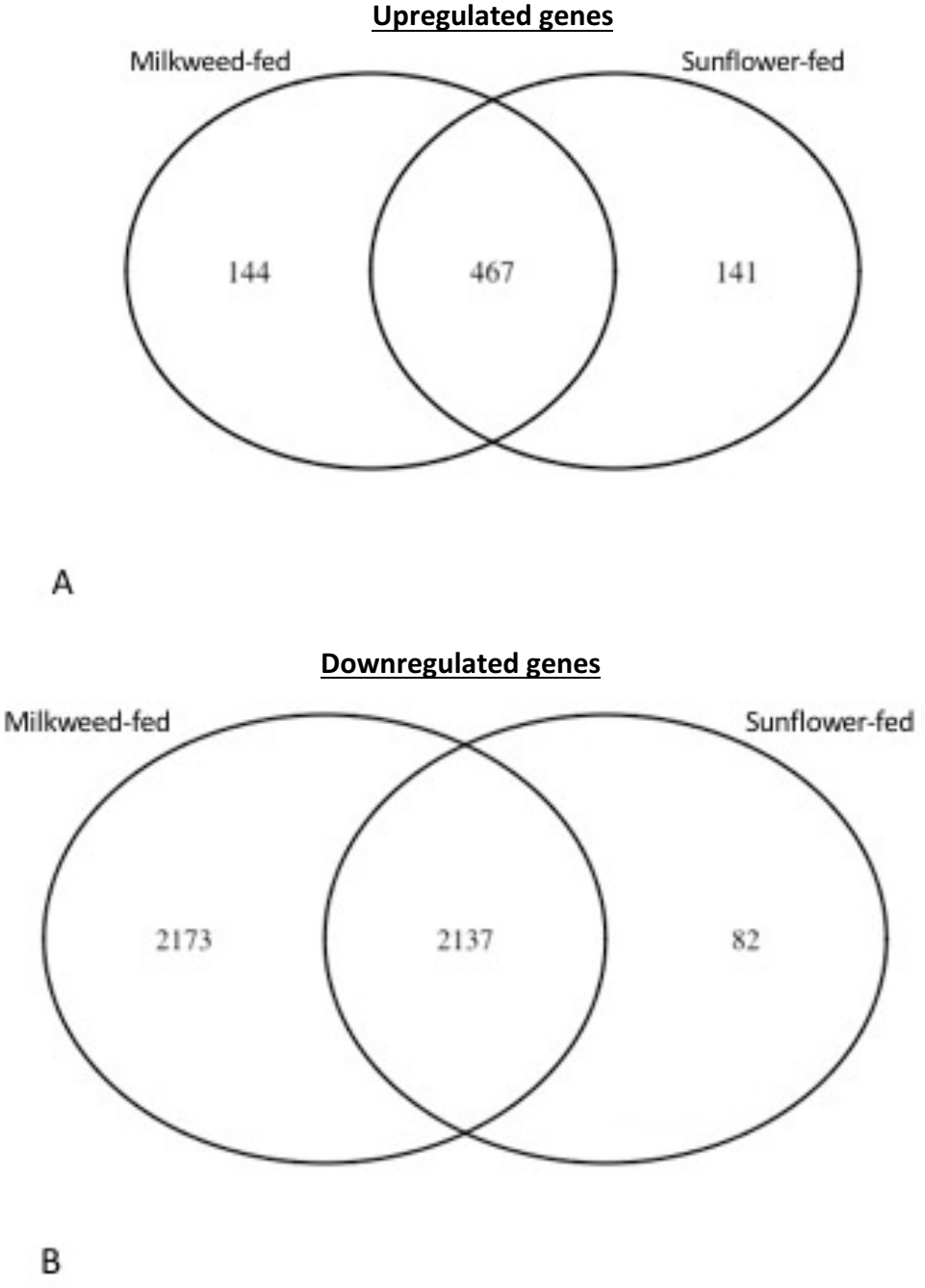

Figure 3.2. Comparison of up- and down regulated genes between diets. Number of (A) upregulated genes and (B) downregulated genes found in each diet, milkweed and sunflower seeds. All genes represented here are significant with a p-value < 0.05.

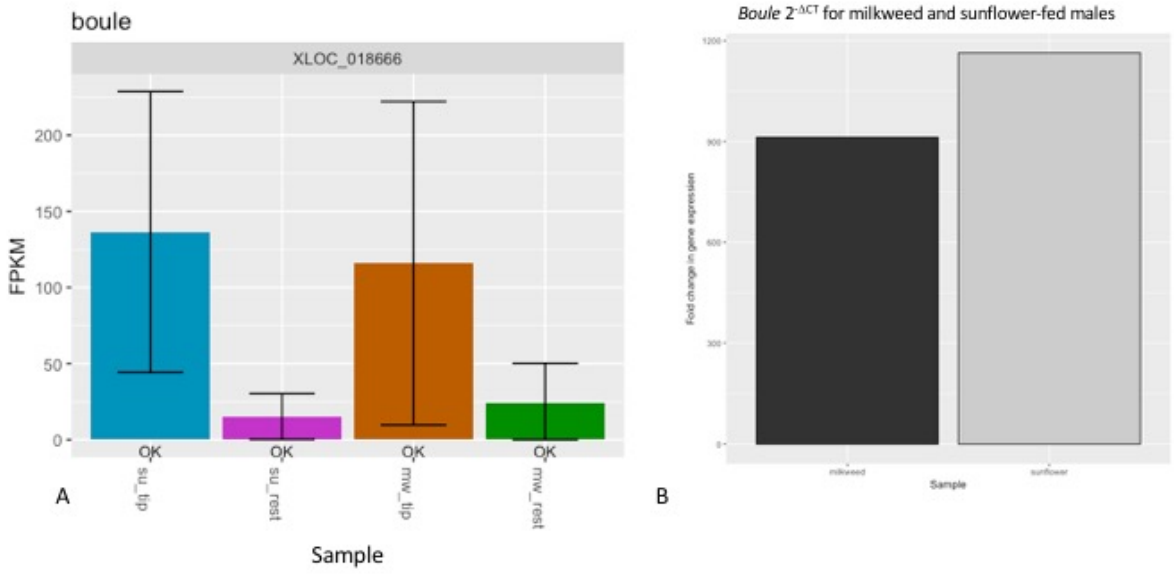

Figure 3.3. *boule* is upregulated in testis distal tips. (A) Fragment per kilobase million (FPKM) counts for each sample and the (B) $2^{-\Delta CT}$ measurements for *boule*. The FPKM (A) shows the relative amount of expression per sample; error bars represent the confidence interval for each sample. There is a trend for there to be more transcripts in the sunflower distal tip than in the milkweed distal tip, as well as compared to the sunflower and milkweed proximal rest. The $2^{-\Delta CT}$ values (B) show more transcripts in sunflower-fed males than milkweed-fed males.

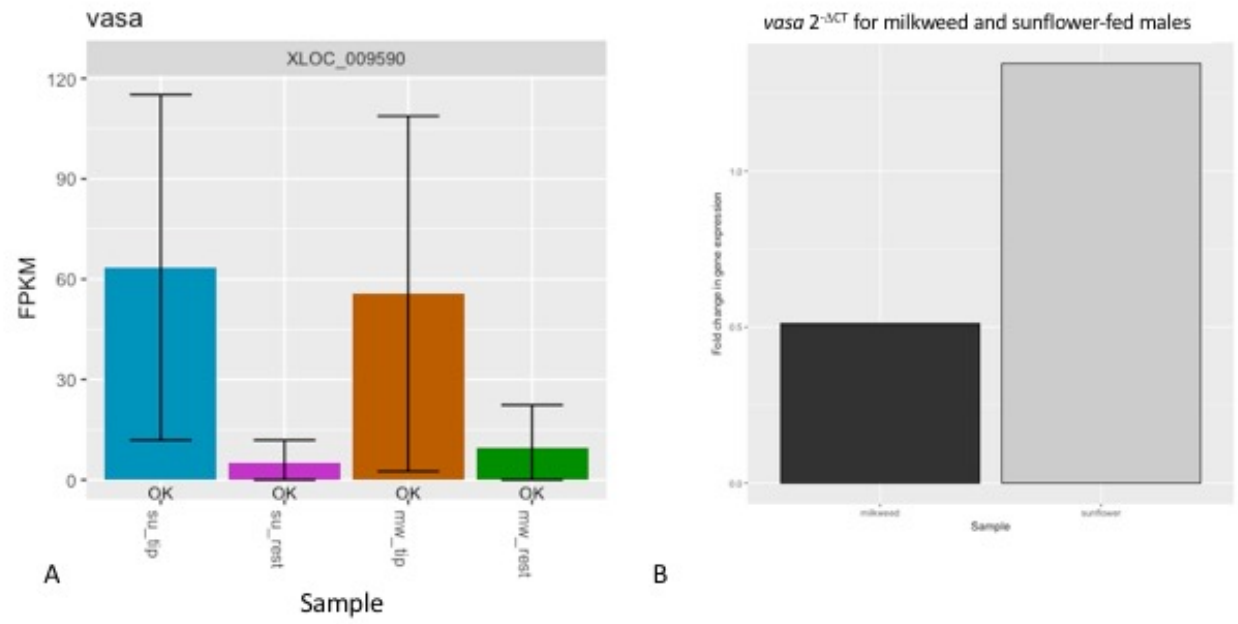

Figure 3.5. *vasa* is upregulated in distal tips of testis. (A) Fragment per kilobase million (FPKM) counts for each sample and the (B) $2^{-\Delta CT}$ measurements for *vasa*. The FPKM (A) shows the relative amount of expression per sample; error bars represent the confidence interval for each sample. Overall, there are more transcripts in the distal tips of both diets as compared to the sunflower and milkweed proximal rest. There are more transcripts in sunflower-fed males as compared to milkweed-fed males, according to the $2^{-\Delta CT}$ data (B), however it is not a large difference.

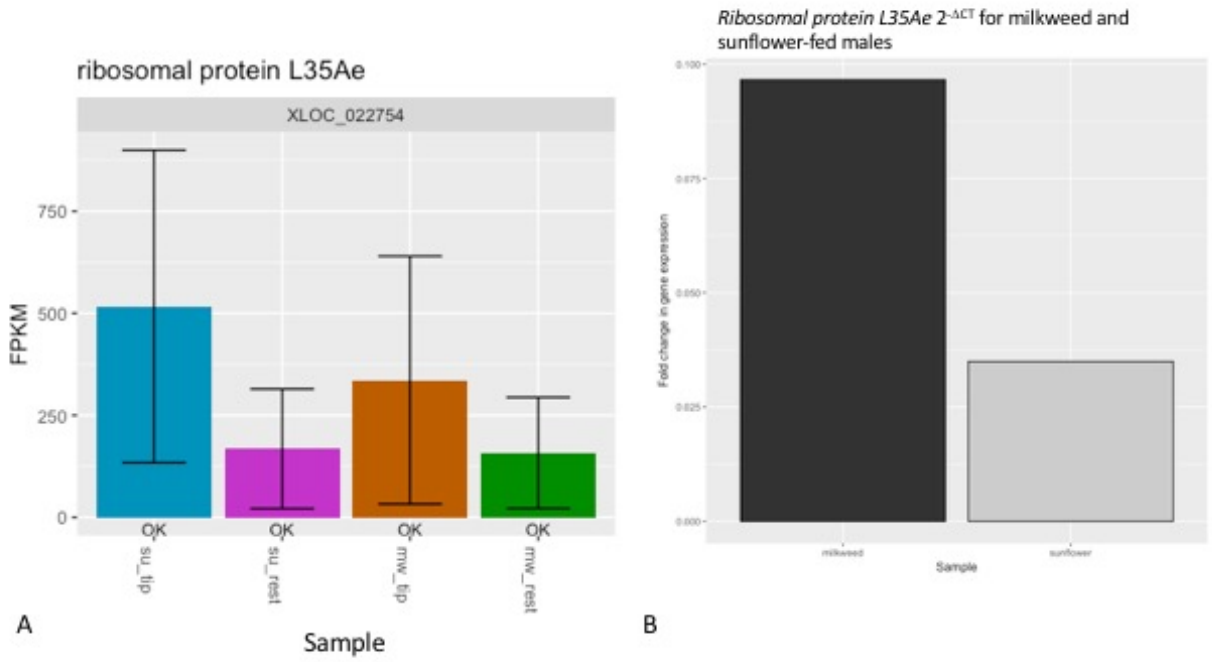

Figure 3.6. *ribosomal protein L35Ae* is more abundant in testis distal tips. (A) Fragment per kilobase million (FPKM) counts for each sample and the (B) $2^{-\Delta CT}$ measurements for *ribosomal protein L35Ae*. The FPKM (A) shows the relative amount of expression per sample; error bars represent the confidence interval for each sample. There is a trend for there to be more transcripts in the sunflower distal tip than in the milkweed distal tip, as well as compared to the sunflower and milkweed proximal rest. The difference in $2^{-\Delta CT}$ values (B) are very small between the milkweed and sunflower-fed males.

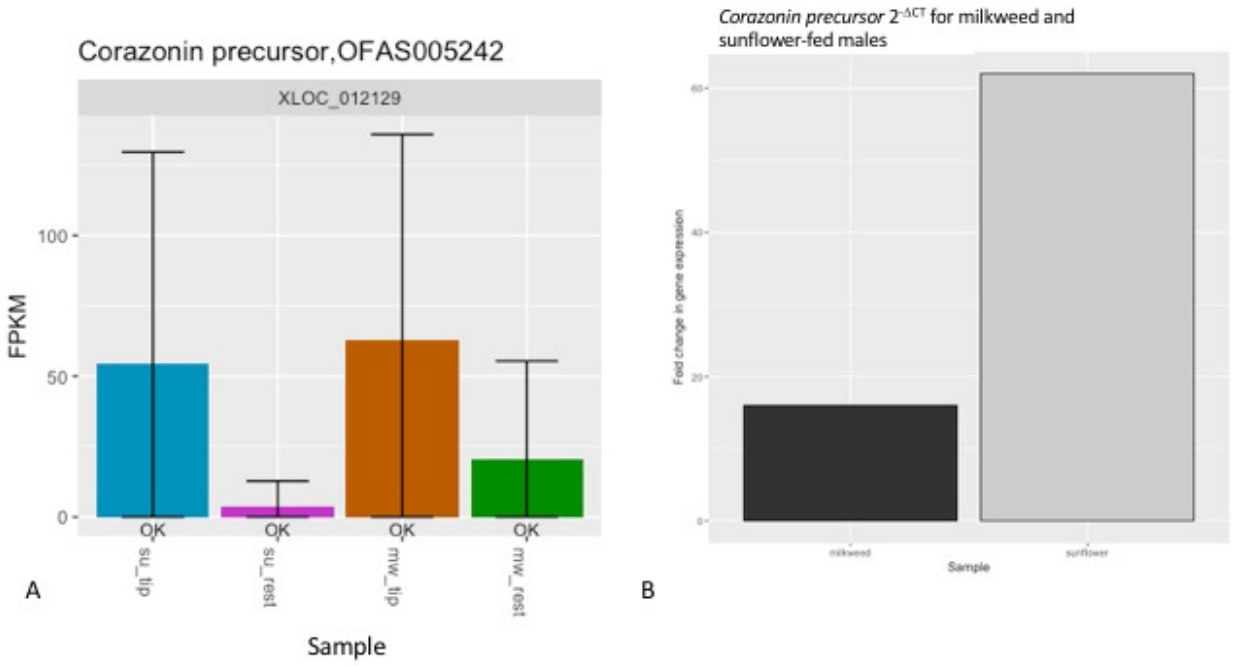

Figure 3.7. *corazonin precursor* is upregulated in distal tips of testis. (A) Fragment per kilobase million (FPKM) counts for each sample and the (B) $2^{-\Delta CT}$ measurements for *corazonin precursor*. The FPKM (A) shows the relative amount of expression per sample; error bars represent the confidence interval for each sample. There is a trend for there to be more transcripts in the distal tip of both diets than in the sunflower and milkweed proximal rest. The $2^{-\Delta CT}$ values (B), show higher gene expression in sunflower-fed males than milkweed-fed males.

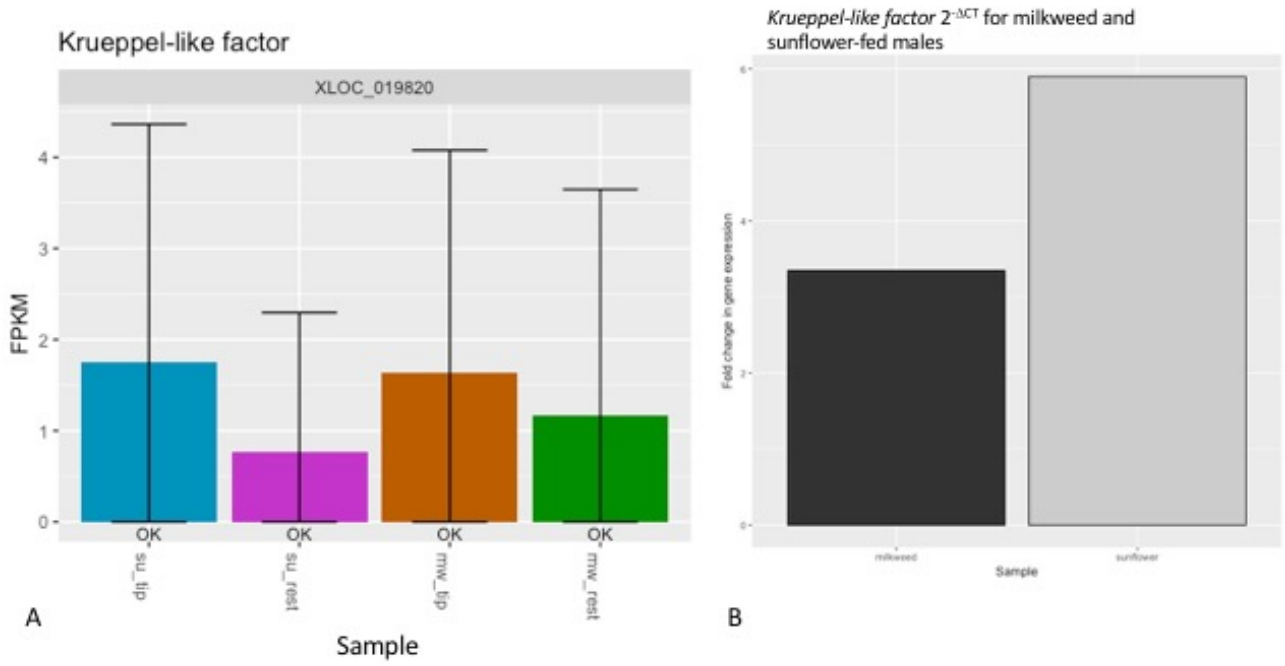

Figure 3.8. *krueppel-like factor* is not favored in one treatment over another. (A) Fragment per kilobase million (FPKM) counts for each sample and the (B) $2^{-\Delta CT}$ measurements for *krueppel-like factor*. The FPKM (A) shows the relative amount of expression per sample; error bars represent the confidence interval for each sample. None of the samples are favored over any of the others. The $2^{-\Delta CT}$ values (B) are close to each other, and have a slight lean towards sunflower-fed males.

Chart 3.1. Primers used for quantitative PCR on *Oncopeltus fasciatus* testes.

Primer Name	Primer Sequence (5' – 3')
vasa_1_for	CTGTTGCTCCTCAGGTTATT
vasa_1_rev	CATTAAGCCTTCCAGGAGTAG
rpL35Ae1forward	CAGGGCGATAACGATGTATG
rpL35Ae1reverse	GGGTTTCAGCAAGGATGAG
boule1forward	CCACCTCTTCCTTTCAGTTC
boule1reverse	CTGGAGGTGCAACATAGTATT
krueppel-like1forward	CTTCTGAACTCTGTCTGGAAC
krueppel-like1reverse	GTCAACCTAGTTGGCCTTATC
hsp83_1forward	GCTCAAGCACTTCGAGATAG
hsp83_1reverse	GGGTCTTCCAGAGCAAATC
actin1forward	GGTAGAAAGAGAAGCAAGGATAG
actin1reverse	GACATCAGGAAGGACTTGTATG
tektin-1_1forward	TCAGGAAGAGTGGCTTCT
tektin-1_1reverse	GACTCTCTGGTGTCTTGTATTC
Of_CZN_RT#1_Forward	TACCTTCTCTGCGAGCTCTA
Of_CZN_RT#1_Reverse	CTATGTCCACGGGAATAGGTTC
Of_CZN_RT#3_Forward	GATTTGGGTCCGCCAGTT
Of_CZN_RT#3_Reverse	TACTGGAAGGTCTGGGAGAG
GAPDH1forward	CGAAGCTGGCCGATAAAT
GAPDH1reverse	AGACATCTCTGAGAGGGTTAG

CHAPTER 4

DISCUSSION AND CONCLUSIONS

My research furthered the understanding of the life history of *Oncopeltus fasciatus* by examining the proximate and ultimate mechanisms by which the reproduction-longevity trade-off occurs. As much of this research has been conducted in *C. elegans* and *D. melanogaster*, this represents a new system in which to view life history in insects, as *D. melanogaster* is a highly derived and holometabolous insect and *C. elegans* is a nematode. *O. fasciatus* is hemimetabolous (Introduction) and is an arthropod, like *D. melanogaster*, and therefore is useful for comparisons.

Male *O. fasciatus* fed a milkweed seed diet choose to reproduce, or put resources into reproduction, at the expense of longevity, where males fed sunflower seeds live longer and put less resources into reproduction (Attisano et al. 2012). My first study looked at this further and then asked at what age are the males more fertile, and is the sperm high quality or high in number, and looked at the rate of spermatocyst division. We showed that milkweed-fed males had higher fertility than sunflower-fed males at both ages and there was no interaction between age and diet and that old milkweed-fed males had significantly greater numbers of sperm stored in their seminal vesicles than old sunflower-fed males, but that the diet did not affect the quality (i.e. viability) of the sperm. When looking at the rate of division, we determined that the spermatocysts in both diets were in relative synchrony, and at two weeks old, they were more likely to be found in the M-phase of the cell cycle (Chapter 2). Males fed

milkweed seeds fertilize more eggs since they make more sperm, but as to why they make more sperm, that is a big question, because both milkweed and sunflower fed males are dividing spermatocysts at around the same rate.

We can take this first study and the information gathered and then use it to determine what may be a factor in why there are differences in the fertility between the two diets. I completed a genome-guided transcriptome on the testis to look at these differences. I found that there were differences between the diets, the distal tip, where the germline stem cells are located, and the proximal rest of the testis follicles. It was also determined that there were more differences in gene sets that belonged to germline stem cells than to spermatogenesis related or "other" genes. A few of the genes found that were relevant to our work, and have been studied in GSCs before included boule, vasa, and piwi (Ewen-Campen et al. 2010, 2013). This data can be mined for genes as more genome annotation versions come out from the i5k consortium (Hughes et al. 2015).

Practical Implications

Even though much of this germline stem cell work has been completed in C. elegans and D. melanogaster, there have been a few studies in humans (e.g. Flatt & Heyland 2011). This work shows that even though it may be in insects right now, there is a place for dietary restriction and protein starvation studies. They can show that protein and fatty acid intake can in fact change the fertility or lifespan of a human (e.g. Bustos and Partridge 2017).

Future Work

There are many parts to the project that are unanswered at this point, this is just because the full *O. fasciatus* genome is not annotated yet. As it is in the process of being

annotated, there will be more genes that can be found in this transcriptome data once more versions are released.

Much of the data is a gene that is up- or down-regulated in a diet. Some ways to further this research would be to complete RNA interference on a select few to determine if they are necessary for the act of spermatogenesis or germline stem cell turnover. At this moment, it would be hard to determine which it is affecting unless we find more suitable markers for the germline stem cells in *O. fasciatus*. Ewen-Campen et al. (2013) has used vasa and Fascilin-III, but in our lab, we were unable to use these two as markers, as we were unable to get them to stain the GSCs and the daughter cells in the testis. It is possible that one of the genes found in this screen could be used for this purpose.

Much of the work completed points to a link between the germline and the insulin/insulin-like signaling (IIS) pathway (Gáliková et al. 2011). Although this is a pathway that is complicated and affects almost everything in an insect, it may be something to consider in the future. Possibly, an experiment in which FOXO, which is controlled by insulin, but is in the germline, is knocked down in *O. fasciatus*, like it has been in *D. melanogaster* (McLeod et al. 2010). The results may be similar as the gene is conserved, but it would still lead us toward the *O. fasciatus* germline stem cell maintenance and turnover pathway and possibly towards a connection in the diet-reproduction-longevity trade-off.

Research Limitations

Working with insects can be tricky, and *O. fasciatus* did not want to cooperate at some points in the project. There were mite and mold problems that affected the first project and led to multiple trials of the projects. During the second project, as with some transcriptomes

currently, this one did not have a completed genome to work off, as *O. fasciatus* is part of the insect 5,000 genome project and is in the process of being annotated, and our work is to be included to help with the annotation. This then means that not all the DEGs found could be determined, and that there are more that could have been important but were not identified in our screen.

The trade-off between reproduction, longevity, and diet of an organism has been extensively studied in *D. melanogaster* and *C. elegans* (Harshman & Zera 2007, McLeod et al. 2010, Gáliková et al. 2011, Harvanek et al. 2017), as well as in *O. fasciatus* (Attisano et al. 2012), but not as extensively. Now that the toolkit has been extended to a list of differentially expressed genes that may be available for researchers to study in the testes or to manipulate to determine what may be a leading mechanism behind this trade-off, there is more use for this model system of *O. fasciatus* in the molecular mechanisms behind the reproduction-longevity trade-off.

REFERENCES

Attisano, A., A. J. Moore, and P. J. Moore. 2012. "Reproduction-Longevity Trade-Offs Reflect Diet, Not Adaptation." *Journal of Evolutionary Biology* 25 (5): 873–80. doi:10.1111/j.1420-9101.2012.02476.x.

Bustos, V., and L. Partridge. 2017. "Good Ol' Fat: Links between Lipid Signaling and Longevity." *Trends in Biochemical Sciences* xx. Elsevier Ltd: 1–12. doi:10.1016/j.tibs.2017.07.001.

Ewen-Campen, B., T. E. M. Jones, and C. G. Extavour. 2013. "Evidence against a Germ Plasm in the Milkweed Bug Oncopeltus Fasciatus, a Hemimetabolous Insect." *Biology Open* 2: 556–68. doi:10.1242/bio.20134390.

Ewen-Campen, B., N. Shaner, K. A. Panfilio, Y. Suzuki, S. Roth, & C. G. Extavour. 2011. “The Maternal and Early Embryonic Transcriptome of the Milkweed Bug Oncopeltus Fasciatus.” *BMC Genomics* 12 (1). BioMed Central Ltd: 61. doi:10.1186/1471-2164-12-61.

Flatt, T., & A. Heyland. 2011. “Mechanisms of Life History Evolution.”

Gáliková, M., P. Klepsatel, G. Senti, and T. Flatt. 2011. “Steroid Hormone Regulation of C. Elegans and Drosophila Aging and Life History.” *Experimental Gerontology* 46 (2–3): 141–47. doi:10.1016/j.exger.2010.08.021.

Harshman, L. G., and A. J. Zera. 2007. “The Cost of Reproduction: The Devil in the Details.” *Trends in Ecology and Evolution*. doi:10.1016/j.tree.2006.10.008.

Harvanek, Z. M., Yang Lyu, C. M. Gendron, J. C. Johnson, S. Kondo, D. E. L. Promislow, and S.D. Pletcher. 2017. “Perceptive Costs of Reproduction Drive Ageing and Physiology in Male Drosophila.” *Nature Ecology & Evolution* 1 (6): 152. doi:10.1038/s41559-017-0152.

Hughes, D. S. T., S. Koelzer, K. A. Panfilio, and S. Richards. 2015. “Oncopeltus Fasciatus Genome Annotations v0.5.3.” Ag Data Commons. doi:http://dx.doi.org/10.15482/USDA.ADC/1173237.

McLeod, C. J., L. Wang, C. Wong, and D. L. Jones. 2010. “Stem Cell Dynamics in Response to Nutrient Availability.” *Current Biology : CB* 20: 2100–2105.